A THESIS

ENTITLED

KALLIKREINS AND KININS IN HEALTH AND DISEASE

BY

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ABSTRACT OF THESIS (Regulation 7.9)

Kinins are potent vasodilator peptides which increase capillary permeability, stimulate prostaglandin synthesis, and when infused intrarenally cause natriuresis and diuresis. Kinins are formed from kininogens by plasma or glandular kallikreins. Plasma kallikrein circulates as an inactive precursor, and interacts with coagulation, fibrinolytic and complement systems. Glandular kallikreins are found in salivary glands, pancreas and kidney. Previous studies found renal kallikrein only in distal tubular cells. Activity of the renal kallikrein-kinin system is inferred from urinary kallikrein excretion rate (UKall).

Methods are described for measurement of plasma prekallikrein and urine kallikrein concentration, using specific synthetic tripeptide chromogenic substrates.

In normal man, UKall increased promptly in response to saline infusion, correlating with increased sodium and water excretion. The ratio of plasma renin activity (PRA) to UKall was highly correlated with urinary sodium excretion (UNaV) (r-0.91). This correlation was also highly significant in normal man during a 9 hour clearance study under basal conditions (r-0.51).

Histological and immunocytochemical studies were performed to characterise the granular glomerular peripolar cell. These cells were found to contain kallikrein in secretory granules, and to lie in close apposition to renin-containing cells.

UKall, factored for glomerular filtration rate, was increased in patients with chronic renal failure. UKall fell in patients given captopril, in association with an increase in plasma creatinine. In patients with nephrotic syndrome (NS), UKall was raised, and there was increased glandular kallikrein activity in plasma. A role for kallikreins and kinins in the pathogenesis of NS is proposed. The PRA/UKall ratio correlated with UNaV (r-0.53). UKall, measured under clearance study conditions, was low in most patients with hepatic cirrhosis, particularly those with ascites. PRA/UKall was a strong correlate with UNaV (r -0.80).

In an ovine model of acute renal failure (ARF) due to sepsis, reciprocal changes in PRA and UKall were observed, correlating with increased plasma catecholamines. PRA/UKall correlated with UNaV (r-0.82) and plasma albumin (r-0.82). This "sodium-retaining" ARF may be due to neuro-endocrine mechanisms, including kallikrein and renin. Acute euvolaemic lowering of plasma protein concentration in sheep by plasmapheresis, did not affect UKall, PRA, or sodium excretion.

The results support the concept that the renal kallikrein-kinin system is natriuretic and diuretic. Kallikrein released from PPCs may influence glomerular and tubular function. Relative activity of the renin-angiotensin and kallikrein-kinin systems appears to be an important determinant of renal function, and of sodium excretion in particular, in both health and disease.

Declaration

I declare that all the work described herein was carried out by myself, in the Department of Medicine and Medical Renal Unit, Royal Infirmary, Edinburgh, and the Department of Medicine, University of Western Ontario at Victoria Hospital, London, Ontario, between 1984 and 1988. Some of the work was done in collaboration with colleagues, as detailed below.

Chapter 3 - Dr ML Watson, Dr Anne T Lambie.

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CHAPTER 1 INTRODUCTION

1.1. The existence of kallikreins and kinins was probably first recognised in 1906, when Abelous and Bardier described the hypotensive properties of human urine when injected into dogs (Abelous and Bardier, 1906). The chemical properties of the active principle in this phenomenon corresponded to later descriptions of kallikreins and kinins, and the International unit for kallikrein was until recently standardised in relation to the fall in blood pressure induced by 5 ml of pooled normal human urine (Frey, Kraut and Werle, 1950). This paper gave the first indication that the kidney generates vasodepressor compounds. 80 years later, the significance of this observation in relation to human disease remains only marginally less obscure.

The basic chemistry of the kallikreins was defined in the 1930s by Frey, Haut and others (Kraut, Frey and Werle, 1930). Subsequently, knowledge of the structure and function of these compounds has accumulated rapidly (Levinsky, 1979; Mills, 1980; Mills, 1982; Scicli and Carretero, 1986; Fuller and Funder, 1986).

1.1.1. Kallikreins

Kallikrein is the generic term for enzymes which generate kinins from kininogen precursors. Kallikreins are widely distributed, both phylogenetically (kallikreins are the active principle in many wasp stings) and in human tissues (Pisano, 1975). An important principle concerns the separation of plasma kallikrein from other types,

collectively known as glandular kallikreins. The fundamental differences between these types of enzyme are shown in Table 1. The most important sources of glandular kallikrein are the pancreas, submandibular glands, and kidney.

	Glandular kallikrein	Plasma kallikrein
Molecular weight	24,000 - 44,000	100,000 - 120,000
Isoelectric point	3.5 - 4.4	8.0 - 8.5
Substrate	LMWK and HMWK	HMWK
Inhibited by SBTI	No	Yes
Normally found as	Active/inactive	Inactive(prekallikrein)
Physiological inhibitors	aprotonin	C1-esterase inhibitor α1-proteinase inhibitor Antithrombin III-heparin

Abbreviations: LMWK - low molecular weight kininogen; HMWK - high molecular weight kininogen; SBTI - soya bean trypsin inhibitor.

Table 1 Characteristics of glandular and plasma kallikreins

1.1.2. Kinins

The first kinin to be recognised, bradykinin, was so named because it produced a slow contraction of uterine smooth muscle in a bioassay preparation. It is a nonapeptide, and the decapeptide, lysyl-bradykinin, has similar actions. Kinins have a wide range of biological effects. which vary between tissues and between species (Regoli and Barabe, 1980). The following properties are relatively consistent, however.

1. Vasodilation. In vitro, kinins produce different

effects on vascular tone in different preparations, but in vivo exert a powerful vasodilator effect on capillary vessels and arterioles. Bradykinin can antagonise the vasoconstrictor action of Angiotensin II at equimolar concentrations (Regoli, Barabe and Park, 1977). Infusion of bradykinin into the renal artery increases renal blood flow (Barraclough and Mills, 1965), an effect which is only partially blocked by indomethacin (McGiff et al, 1976; Vio, Bednar and McGiff, 1983). Kinins reduce the effect on renal function of stimulation of the renal sympathetic nerves (Inokuchi and Malik, 1984).

2. Increased capillary permeability. Kinins have long been considered to be important mediators of inflammation, and local reactions to injected kinins include erythema, pain, oedema, and increased capillary protein leak (Marceau et al, 1983).

3. Stimulation of prostaglandin synthesis. Kinins are widely used by physiologists to stimulate release of arachidonic acid from cell membranes, via activation of Phospholipase A2 (Nasjletti and Malik, 1981). Some, but not all, of the actions of kinins are mediated by prostaglandins as second messengers (McGiff, 1980; Mullane and Moncada, 1980; Kramer et al, 1983).

Other actions of kinins include bronchoconstriction, control of sperm motility, stimulation of cell proliferation, stimulation of catecholamine release from the adrenal medulla, release of histamine from mast cells, and

facilitation of insulin-mediated glucose transport (Regoli and Barabe, 1980).

1.1.3. Kinin receptors

Stewart, Regoli and others have worked to clarify the nature of these receptors (Stewart, 1968; Vavrek and Stewart, 1987; Regoli, Marceau and Lavigne, 1981). It is clear that at least two types exist. The most important is the B2 receptor, which is widespread in its distribution, and responds to both bradykinin and kallidin. It has recently been found to be antagonised selectively by a highly substituted kinin analogue, B3824 (Vavrek and Stewart, 1987). The B1 receptor has uncertain significance in physiology, and appears to be confined to vascular tissue. It is antagonised by [Leu-OMe8] des-Arg9-Bradykinin . Trace quantities of bacterial lipopolysaccharide increase the sensitivity of tissues to the B1 agonist, des-Arg9-Bradykinin, and Kininase 1 produces kinin fragments which have B1 agonist properties and prolonged plasma half-lives. These mechanisms may allow potentiated effects of kinins during endotoxaemia (Regoli and Barabe, 1980). Post-receptor events involve an increase in intra-cellular cAMP, by either increased adenyl cyclase or decreased phosphodiesterase activity. This stimulates an energy-requiring calcium-binding process, leading to a fall in intra-cellular calcium and muscle relaxation (Bareis et al, 1983). The recent discovery of competitive antagonists of both B1 and B2 receptors may help to answer many of the unresolved questions regarding the actions of kinins.

1.1.4. Inhibitors of the Kallikrein-Kinin systems

The important inhibitors of the plasma and glandular kallikreins are shown in Table 1. The plasma kallikrein-kinin system is normally completely inhibited by an excess of α1-proteinase inhibitor and C1 esterase inhibitor (Schreiber, Kaplan and Austen, 1973). The importance of endogenous inhibitors of glandular kallikreins is less clear, although several tissues, including lung and kidney, are rich in such compounds (Kunitz and Northrop, 1936). The most important pharmacological kallikrein inhibitor, aprotonin, is a polypeptide, molecular weight 6512, which binds to trypsin, plasma kallikrein, plasmin, and glandular kallikreins (Fritz and Wunderer, 1983)(Table 2). It is prepared commercially as a purified extract of bovine lung (Trasylol, Bayer FRG).

Enzyme	Dissociation constant (Ki)
Trypsin	6.0 x 10-14
Glandular kallikrein	9.0 x 10-11
Plasmin	2.3 x 10-10
Plasma kallikrein	3.0 x 10-8
Neutrophil elastase	3.5 x 10-6

Table 2 Specificity of aprotonin

Kinins are rapidly metabolised by Kininase enzymes, so that the plasma half-life of bradykinin is of the order of 15 seconds (McCarthy, Potter and Nicolaides, 1965). The most

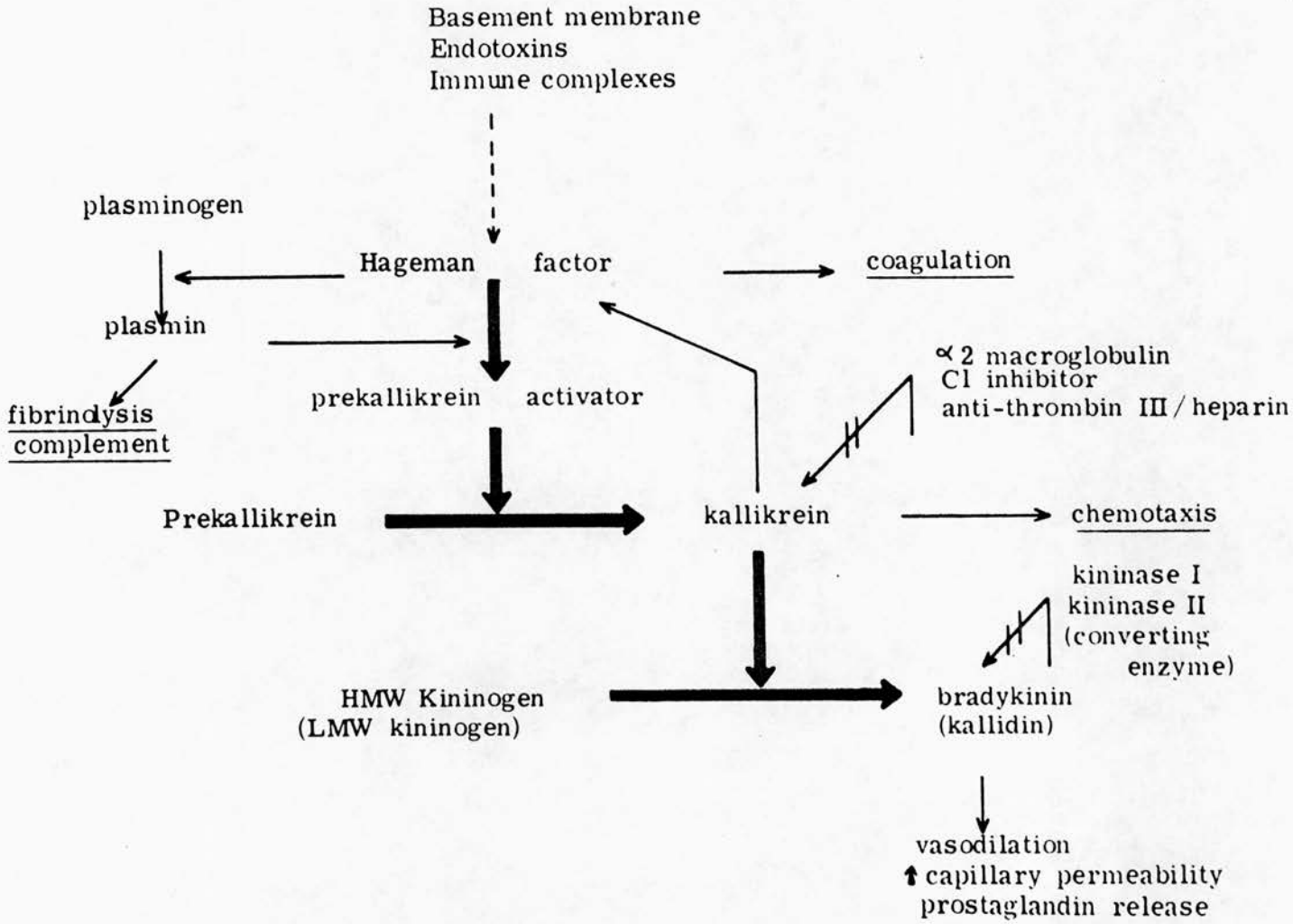
important enzymes are Kininase 1 and Kininase 2, and the main sites of kinin metabolism are the lung and kidney. Kininase 2 is identical to Angiotensin Converting Enzyme (ACE), and ACE inhibitors may potentiate the kallikrein-kinin system by slowing kinin metabolism (Erdos, 1975; Carretero, 1981).

1.2. The plasma kallikrein-kinin system

Components of the plasma kallikrein-kinin system and its important interactions are shown in Fig 1. Plasma kallikrein normally circulates almost entirely as an inactive precursor. prekallikrein, which is synthesised in the liver and has a molecular weight of 130,000 (Kaplan, Meier and Mandle, 1977). Activation of Hageman factor leads to conversion of prekallikrein to kallikrein (M. Wt. 100,000) (Bagdasarian et al, 1974). Hageman factor is also the initiating enzyme of other contact-activated systems in plasma, including blood coagulation, fibrinolysis, and complement activation (Cochrane et al, 1972). Hageman factor is activated by contact with exposed basement membranes, collagen, other negatively charged surfaces, and by bacterial endotoxin (Morrison and Cochrane, 1974). The plasma kallikrein-kinin system has been considered one of several interlocking networks which help to mediate cellular and vascular responses necessary for defence and repair (Fuller and Funder, 1987).

The principal substrate of plasma kallikrein is the high molecular weight kininogen, M. Wt. 200,000, and the main

Fig. 1. The plasma kallikrein-kinin system



product is bradykinin. Kininase 1 is responsible for 90% of the kinin degrading activity of plasma, but Kininase 2 located in the lung is quantitatively more significant, so that 90% of the biological activity of bradykinin is lost during a single passage through the lungs (Erdos, 1976).

1.2.2. Physiology

Because injection of bradykinin lowers blood pressure, a role for the plasma kallikrein-kinin system in the regulation of blood pressure has been proposed (McGiff and Nasjletti, 1976, Margolius, 1980). There is however little evidence for such a mechanism under physiological conditions, and it is difficult to conceive of such an effectively inhibited enzyme system having such a role in the systemic circulation (Waeber et al, 1988). The blood pressure response to bradykinin infusion is in fact very variable, and in some species, bradykinin increases blood pressure, probably by a central effect (Mills, 1979). Indeed it has been suggested that kinins function solely as local tissue autocoids, and that plasma kinin generation only becomes important under pathological conditions, or when kinin degradation is inhibited (Fuller and Funder, 1987). There is good evidence that the hypotensive effect of ACE inhibitors in low-renin hypertensives and in anephric subjects is related to increased plasma kinin concentrations (Overlack, Stumpe and Kuhnert, 1981).

1.2.3. Pathophysiology

Using a radiolabelled enzyme substrate, p-toluene-sulphonyl-L-arginine-methyl ester (TAME), in an activity assay, Colman

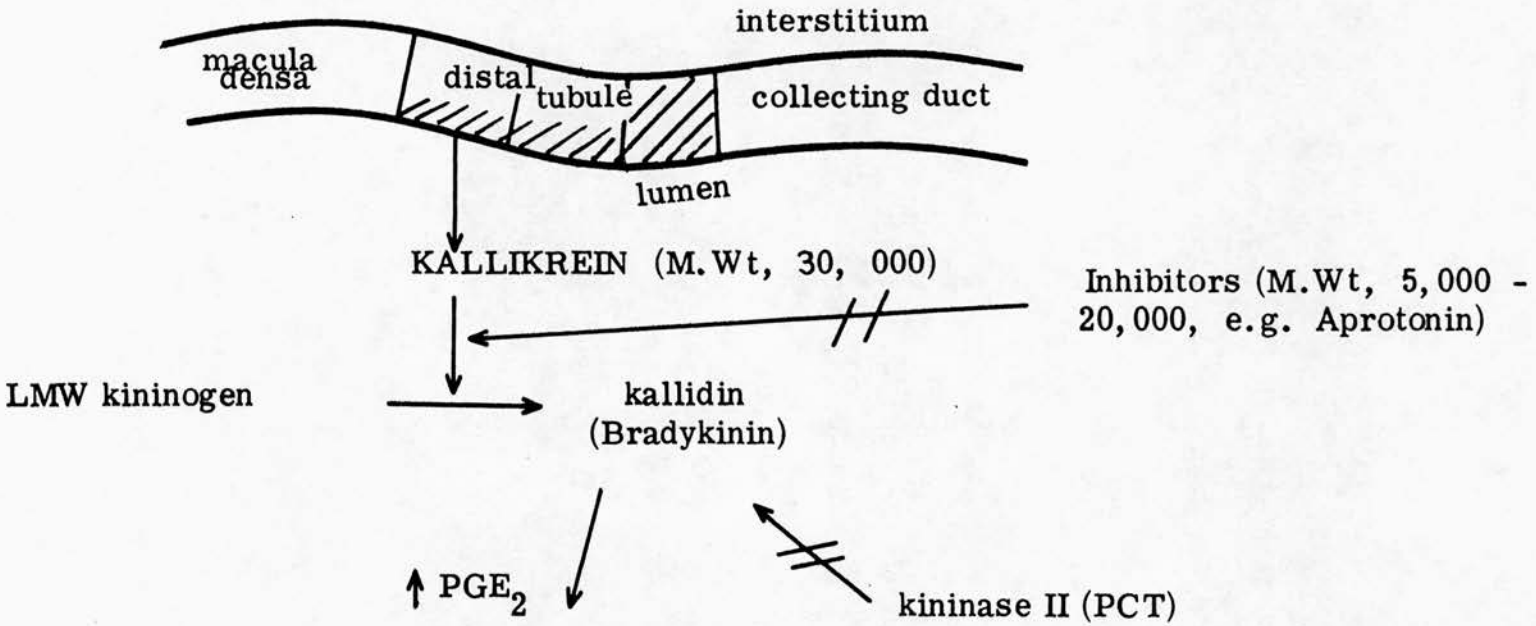
and colleagues, and others, have described abnormalities of the plasma kallikrein-kinin system in a wide range of disease states (Colman and Wong, 1977). In hereditary angioneurotic oedema, deficiency of C1 esterase inhibitor is associated with recurrent episodes of kallikrein-kinin system activation (Ratnoff et al, 1969). In septic shock, reduced plasma concentrations of plasma prekallikrein, increased "kallikrein-like activity", and decreased kallikrein inhibitory activity have been documented, probably as a result of endotoxin-induced Hageman factor activation (Webster and Clark, 1959; Neiss et al, 1968; Mason et al, 1970; O'Donnell et al, 1976; Cumming et al, 1984). Reduced plasma prekallikrein has also been observed by Colman's group in a number of other disease states, including disseminated intravascular coagulation, typhoid fever, hepatic cirrhosis, coronary artery disease, transfusion reactions, and in relation to haemodialysis and extracorporeal bypass. It is unclear in these syndromes whether the reduced plasma prekallikrein reflects kallikrein-kinin system activation or reduced prekallikrein synthesis, and the possible significance of the kallikrein-kinin system abnormalities has not been tested by the use of aprotonin or other more selective inhibitors. The role of the plasma kallikrein-kinin system in human disease therefore remains uncertain, although several provocative experimental observations suggest that it may participate in at least some clinical conditions. Recent studies using

selective kinin antagonists are shedding further light on this problem, eg suggesting a role for kinins in intracranial vascular spasm after cerebral haemorrhage (Heizer et al, 1987; Waeber et al, 1988).

1.3 Renal kallikrein-kinin system

1.3.1 Renal kallikrein is one of several related glandular kallikreins. They are glycoproteins, single polypeptide chains with molecular weights 27-40,000. They belong to a family of enzymes known as serine proteases, because of the presence of a serine residue at the active catalytic site. They act on either HMWK or low molecular weight kininogen (LMWK) - M. Wt. 50,000 - and in general form lysyl-bradykinin (kallidin) (Levinsky, 1979; Mills, 1982; Scicli and Carretero, 1986; Fuller and Funder, 1987). A schematic representation of the renal kallikrein-kinin system is shown in Fig 2. Renal kallikrein has been localised to the luminal cells of the distal convoluted tubule, between the JGA and the cortical collecting duct, in a variety of species including man, and by a number of techniques, including use of cDNA probes (Orstavvik et al, 1976; Scicli et al, 1976; Vio and Figueroa, 1985). The genes regulating kallikrein synthesis, of which there are at least three in the mouse, are located on Chromosome 7 (Mason et al, 1983). The derived mRNA codes for the synthesis of a pre-prokallikrein, which is cleaved to prokallikrein in the endoplasmic reticulum, and then to fully active kallikrein by a trypsin-like cleavage. Although trypsin can effect this

Fig 2. The Renal Kallikrein- Kinin System



conversion in vitro, the factors responsible for activation of prokallikrein in vivo are unclear, as are the means whereby this conversion is regulated (Fuller and Funder, 1987). Prokallikrein and kallikrein exist at both the luminal and basolateral membranes of distal tubular cells, although at the luminal surface the enzyme projects into the tubular lumen, permitting it to function as an ectoenzyme (Chao and Margolius, 1979). Secretion of kallikrein is thought to occur both into the tubular lumen and into the renal interstitium; kallikrein has been detected in both renal lymph and, in trace quantities, in the systemic circulation (De Bono and Mills, 1974, Proud et al, 1983, Vio et al, 1983). Kallikrein in the tubular lumen probably acts primarily on filtered LMWK, although kininogen has been localised to tubular cells and could also be secreted (Pisano, 1983). The proximal tubular brush border is rich in kininase activity, so that any filtered kinin is degraded before reaching the distal nephron (Carone et al, 1976). Similarly, while kallikrein should not be filtered in appreciable amounts by the normal kidney, the proximal tubule has the capacity to reabsorb and metabolise any filtered enzyme (Mills, Paterson and Ward, 1975) . The circumstances therefore exist for the renal kallikrein-kinin system to function as a local paracrine mechanism, independent of the plasma system. The kallikrein enzyme in urine has been shown to be identical to renal tissue kallikrein (Orstavvik et al, 1976).

1.3.2. Physiology of the renal kallikrein-kinin system

Several roles for the renal kallikrein-kinin system in renal physiology have been proposed, some of which are mutually exclusive. Despite a vast body of experimental evidence, no true concensus has emerged. The most important possibilities are outlined below.

1. Natriuresis. It has long been known that infusion of kinin into the renal artery increases sodium excretion (Barraclough and Mills, 1965). Interest in this as a regulatory mechanism was stimulated by Mills, who while working with De Wardener on the original experiments which suggested the existence of a "natriuretic hormone", postulated that the rapidity of the changes in sodium excretion seen in these experiments was only compatible with an intra-renal mechanism, rather than extra-renal synthesis and release of a hormone. In a series of subsequent experiments he accumulated evidence for the participation of renal kallikrein in a "natriuretic cascade", involving interactions with dopamine, prostaglandins, and low molecular weight ATP-ase inhibitors, and inhibited by adrenergic mechanisms (Adetuyibi and Mills, 1972; Mills et al, 1976; Mills, Newport and Obika, 1979; Mills, 1982). This concept is supported by the observed increase in kallikrein excretion in response to acute sodium-loading in dogs (Mills, 1976), and chronic salt loading in rats (Croxatto et al, 1976); the increase in kallikrein excretion during the DOCA escape phenomenon (Bonner et al, 1981); and by the impressive decreases in sodium excretion induced in some

experimental situations by anti-kinin antibodies (Marin-Grez, 1974) and by the kallikrein inhibitor aprotonin (Kramer et al, 1984). The major objection to the hypothesis was that kinins had not been shown by micropuncture to directly influence proximal tubular sodium transport (Stein et al, 1972); this does not however preclude an effect mediated by second messengers in the intact kidney, a primarily distal effect, or an effect on deep nephrons inaccessible to micropuncture. It is also possible that kinins may alter medullary blood flow and the medullary osmotic gradient (Scicli and Carretero, 1986). One study by Kauker suggested that intra-luminal kinins may reduce efflux of sodium from the late proximal tubule (Kauker, 1980).

2. Sodium retention. The uncertainty regarding the role of the renal kallikrein-kinin system is demonstrated by the fact that Marks and Keiser, established workers in this field, proposed as recently as 1983 that kallikrein was a sodium retaining mechanism, which mediated the tubular effects of aldosterone and other mineralocorticoids (Marks and Keiser, 1983). While urinary kallikrein excretion is increased in primary aldosteronism, Bartter's syndrome, and during DOCA administration, it is normal or low in other circumstances where plasma aldosterone is increased, eg 2-kidney 1-clip renovascular hypertension (Geller et al, 1972; Margolius et al, 1974; Halushka et al, 1977; Scicli and Carretero, 1986). Administration of aldosterone itself does not increase kallikrein excretion (Adam and Clappison,

1982). It is therefore possible that the increased kallikrein excretion is a response to mineralocorticoid-induced sodium retention, rather than a direct effect.

3. Facilitates water excretion by antagonising ADH. Most studies have found that water loading increases kallikrein excretion, and in rabbits subjected to independent alterations in sodium and water intake, the principal correlation was between kallikrein excretion and water intake (Mills and Ward, 1975). Bradykinin, in concert with prostaglandins, can antagonise the hydrosmotic actions of ADH on isolated distal tubules and collecting duct (Schuster, Kokko and Jacobsen, 1984). ADH has been shown to stimulate both the release of kallikrein and intra-renal kinin formation in animals (Fejes-Toth, Zahajsky and Filep, 1980). Such a role would obviously be in keeping with the distal location of tubular kallikrein.

4. An intra-renal vasodilator system which antagonises the vasoconstrictor effect of locally generated Angiotensin II. The concept that the renal kallikrein-kinin system and the renin-angiotensin system are parallel and opposing systems was propounded by Mills and others, and is supported by considerable evidence (Mills et al, 1972). Infusion of Angiotensin II into the renal artery increases kallikrein excretion; this coincides with the development of tachyphylaxis to the effects of AII on renal blood flow and sodium excretion (Macfarlane, Adetuyibi and Mills, 1974). Conversely, both renal kallikrein and kinins can stimulate renin release from superfused kidney slices (Arakawa, 1980;

Beierwaltes, Prada, and Carretero, 1985); aprotonin inhibits basal and frusemide-stimulated renin release (Seto et al, 1983). Kallikrein can convert prorenin to renin in vitro, but it is not certain whether this process is important in vivo (Sealey et al, 1978). In the isolated perfused kidney, kallikrein and renin release change in parallel in response to changes in perfusion pressure and renal blood flow (Bevan, MacFarlane and Mills, 1974). Evidence therefore exits for a functional link between these two systems, although the significance of this relationship in the regulation of renal function is not clear.

5. "Passive washout". Urinary kallikrein excretion rate increases in response to most experimental manoeuvres which increase urine flow, and after repeated stimuli such as mannitol infusions, the increase becomes progressively less marked (Bonner et al, 1981). This has been interpreted as indicating passive washout of kallikrein from tubular cells (Seeber, Vila and Catanzaro, 1982). Such a phenomenon is also seen with most other urine enzymes. It does not however preclude a functional role for intra-renal kallikrein , particularly if it acts to facilitate water excretion, as described above. The reduction in urine flow in volume-expanded rats given aprotonin, and the potentiation by aprotonin of the effect of AVP on urine flow, argue in favour of such a functional role (Kramer et al, 1984). Fejes-Toth et al have recently clearly shown that urinary kallikrein excretion is not flow-dependent in

the conscious rat (Fejes-Toth et al, 1983).

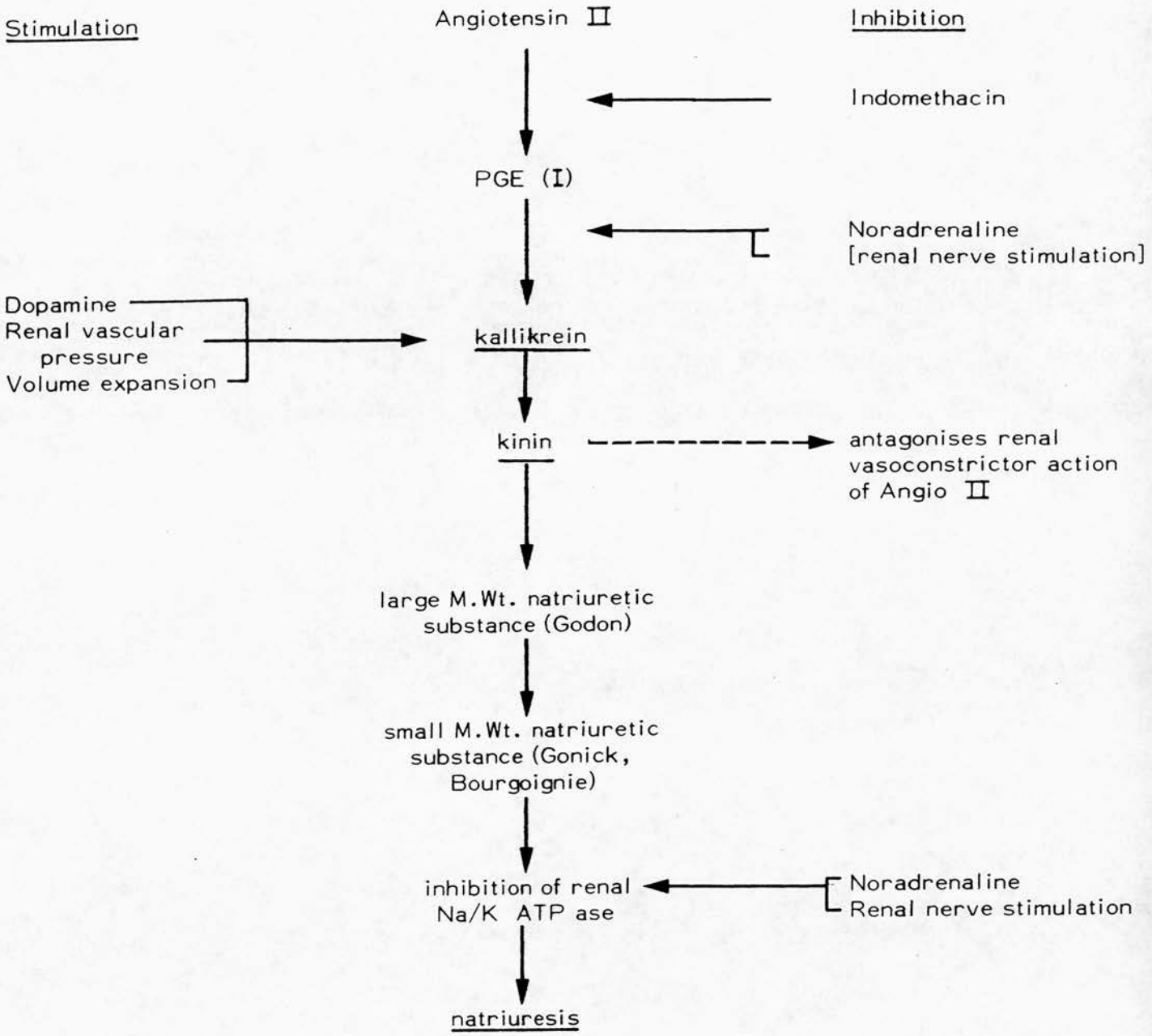
The renal kallikrein-kinin system therefore appears to interact in a complex fashion with other intra-renal neuroendocrine mechanisms. Despite extensive study, the precise nature of these interactions and their importance in regulating renal function have not been fully established.

1.3.3. Pathophysiology

Reduced urinary kallikrein excretion in essential hypertension was described in 1934 (Elliot and Nuzum, 1934), and has since been confirmed; the ratio of active to inactive kallikrein is also abnormally low in essential hypertension (Leiberthal et al, 1983). Kallikrein excretion is lower in families with high blood pressure, and lower in black than in white children (Zinner et al, 1978). The reduced kallikrein excretion is not related to aldosterone or plasma renin activity (Lawton, 1980). In spontaneously hypertensive rats, reduced active kallikrein excretion is present before and during the development of hypertension (Geller et al, 1975). Kallikrein excretion is also reduced in both clinical and experimental renovascular hypertension, and in the hypertension of chronic parenchymal renal disease (Marin-Grez, 1982; Margolius et al, 1974; Adetuyibi and Mills, 1972; Abe et al, 1981; Mitas at al, 1978). It has been reported that oral administration of kallikrein lowers the blood pressure in essential hypertension (Overlack et al, 1979), and a stimulatory effect on the renal kallikrein-kinin system has been thought responsible for the antihypertensive effect of some drugs, eg thiazide diuretics

(O'Connor et al, 1977). Urinary kallikrein excretion is increased in some, but not all, patients with primary aldosteronism, and in Bartter's syndrome; this is probably secondary to other hormonal changes (Scicli and Carretero, 1986). In Bartter's syndrome, kallikreins and kinins may be involved in the vascular insensitivity to Angiotensin II, which is substantially reversed by aprotonin (Rodriguez-Portales et al, 1985). Urinary kallikrein excretion falls during normal pregnancy, and is lower in hypertensive pregnancy (Millar and Campbell, 1986). Urinary kallikrein excretion is elevated in the syndrome of inappropriate secretion of ADH (Shiigia et al, 1983). Excretion of both active and inactive kallikrein is reduced in uninephrectomised kidney donors, and is lower still in transplant recipients (Spragg et al, 1985). It is also low in patients with cadmium toxicity (Iannaccone, Porcelli and Boscolo, 1983); in both situations, this may reflect loss of functional renal tissue mass. Patients with hepatic cirrhosis and renal impairment have been described as excreting less kallikrein than other cirrhotic patients, despite secondary aldosteronism (Zipser et al, 1981; Perez-Ayuso et al, 1984).

Overall, while abnormalities of the renal kallikrein-kinin system have been recognised in a number of disease states, it is unclear in most situations whether these changes are primary or secondary, and whether they have functional significance.



From Mills, I.H., Quart. J. Exp. Physiology, 1982: 67; 393-399.

Figure 3. The natriuretic cascade

CHAPTER 2 METHODS

Study of kallikreins and kinins has been beset by methodological problems, and there remains controversy concerning the optimal methods for assessing activity of both the plasma and renal kallikrein-kinin systems.

2.1. Plasma kallikrein-kinin system

The original studies of this system were based on bioassays using guinea-pig or cat ileum, or, most commonly, the rat uterus, to measure circulating kinins (Erspamer, 1948). However, several other agents were shown to be active in these assays, eg 5-HT, angiotensin, acetyl choline and prostaglandins. Vane and colleagues tried to improve the specificity by using multiple superfused tissues in sequence, but even using such a complex system, the results are influenced by peptides such as Substance P and cholecystokinin (Ferreira and Vane, 1967; Regoli and Barabe, 1980). These assays are not sufficiently sensitive to measure kinins in normal blood. They have been used to estimate plasma kininogen, after in vitro activation of kallikrein to convert kininogen to kinin. A fall in kininogen is taken to indicate activation of the plasma kallikrein-kinin system. However, blood contains a high concentration of kininogen (4-12 ug/ml) in relation to the kinin concentration (pg/ml) even in pathological states, so that a detectable change in plasma kininogen may well be absent (Regoli and Barabe, 1980).

Several groups have developed radioimmunassay (RIA) methods

for measurement of plasma kinins (Talamo and Goodfreind, 1979). The principal problems are the weak immunogenicity of kinins, the tendency of anti-kinin antibodies to cross-react with kininogen and with kinin fragments, spontaneous kinin formation after blood sampling, and the rapid degradation of kinins by plasma kininases. Carretero and colleagues have developed an assay which yields plasma kinin concentrations in the pg/ml range and high recoveries, but the sampling technique involves taking blood directly into absolute alcohol, and is impractical in many types of study (Scicli, Diaz and Carretero, 1983).

An alternative is to measure the plasma prekallikrein concentration. An RIA technique has been described (Saito, 1979), but a more common method is to activate the prekallikrein in the sample completely, then measure activity against a substrate of plasma kallikrein (Webster and Peirce, 1961). Kallikrein is the principal esterase in plasma under alkaline conditions, and ability to hydrolyse substrates such as TAME at a high pH has been used in many studies (Colman and Wong, 1977). An advantage of this approach is that spontaneous "kallikrein-like" activity and the kallikrein inhibiting activity of plasma can also be measured, giving an overall assessment of plasma kallikrein-kinin system activity (Mason et al, 1970). The principal disadvantage has been the limited specifity and sensitivity of the assays. Recently, development of synthetic tripeptide chromogenic substrates, against which kallikrein has

relatively high specific activity, has significantly improved these techniques (Friberger et al, 1979; Gallimore and Friberger, 1982).

2.2. Renal kallikrein-kinin system

The difficulties in measurement of plasma kinins referred to above are equally applicable to urine measurements. Bladder urine contains both kinin-forming and kininase activity, and most authorities believe that direct urine collection from ureteric catheters is the only acceptable method (Shimamoto at al, 1978; Talamo and Goodfreind, 1979; Scicli, Diaz and Carretero, 1983). Again this is impractical for the majority of studies.

As a result, most workers have assessed activity of the renal kallikrein-kinin system by measurement of urinary kallikrein excretion. This can be done using a kinin RIA to measure kininogenase activity (Carretero et al, 1976); by measurement of urinary alkaline esterase activity, against substrates such as TAME (Feidler, 1979); by estimating activity against synthetic tripeptide chromogenic substrates of urinary kallikrein (Amundsen et al, 1979); or by direct RIA for urinary kallikrein (Oza et al, 1981). The latter will measure inactive and inhibitor-bound kallikrein, in addition to the active enzyme. This is useful in certain contexts, and permits calculation of the ratio of active to inactive kallikrein - normally in the region of 50%. However, the factors which activate prokallikrein in vivo are unclear, and the physiological significance of this ratio is unknown (Leiberthal et al, 1983). There is no

evidence that the ratio of active to inactive kallikrein alters as urine passes down the nephron, and measurement of enzyme activity in urine seems most likely to reflect intrarenal events (Ryan et al, 1979; Bonner et al, 1984). Several studies have failed to find a correlation between urinary kinin excretion and urinary kallikrein excretion (Scicli, Diaz and Carretero, 1983; Weinberg et al, 1987). This is perhaps not surprising in view of the problems associated with both estimations, and particularly with kinin measurements. Urine kallikrein estimations are technically simple and reproducible, and appear to give useful and consistent results in a wide range of experimental and clinical situations (Bonner and Marin-Grez, 1981). However, the extent to which either urinary kinin or kallikrein estimations reflect the activity of the intra-renal kallikrein-kinin system remains unproven (Fuller and Funder, 1987).

2.3. Assays used in this project

For the purposes of these studies, the recently developed synthetic tripeptide chromogenic substrate kallikrein assays referred to above were chosen, on the grounds of convenience, cost, reasonable specificity and sensitivity, reproducibility, and good correlation with the results of other more complex assays (Gallimore and Friberger, 1982; Amundsen et al, 1979; Bonner and Marin-Grez, 1981).

2.3.1. Plasma prekallikrein

This was measured by the method of Gallimore and Friberger

(1982). For all blood studies, citrated plasma (1 part 0.1M sodium citrate, 9 parts blood) was used. Blood was taken with a minimum of stasis into plastic tubes, to avoid contact activation of Hageman factor by glass. After centrifugation at room temperature, plasma was removed with plastic pipettes and stored in plastic tubes at -20°c. A standard plasma pool was prepared using samples from 20 healthy donors. Plasma samples were thawed at 20°c prior to assay, and were assayed within 4 hours.

Prekallikrein activator was obtained from AB Kabi Diagnostica; it is an ellagic acid-phospholipid type containing FXII and HMW kininogen, and allows complete activation of prekallikrein even in Factor II and kininogen-deficient plasmas.

Chromogenic substrate for plasma kallikrein was obtained from AB Kabi Diagnostica; S-2302 (H-D-Pro-Phe-Arg-pNA) 2 mmol/l was prepared by dissolving 25 mg in 20 ml distilled water.

Buffer - a Tris-HCl buffer, 0.05 mol/l, pH 7.9, was prepared by dissolving 7.88 g Tris-HCl in 800 ml distilled water and titrating to pH 7.9 with 1M NaOH.

Plasma dilutions were made in plastic tubes, and spectrophotometric determinations were performed in siliconised semimicro cuvettes, using a Gemstar spectrophotometer (Electro-Nucleonics Int Ltd, Breda, Holland).

Determination of prekallikrein

1. Factor XII ----contact activation----->Factor XIIa

2. Prekallikrein ------FXIIa------>Kallikrein

3. H-D-Pro-Phe-Arg-pNA +H2O----kallikrein---->Peptide + pNA

The normal plasma pool was diluted with buffer to give standard values-

Standard (%)	Plasma (µl)	Buffer (µl)
125	50	2400
100	50	3000
The 100% standard was then diluted as shown		
75	300	100
50	200	200
25	100	300
0	0	400

Test plasma were diluted as for the 100% standard.

Method Into each cuvette, at 37°c, was pipetted -

- 200 µl prekallikrein activator (at 37°c)
- 200 µl plasma dilution
- After 2 mins incubation at 37°c, 200µl S-2302 was added
- After 2 mins incubation at 37°c, 200µl 50% acetic acid was added to stop the reaction, and the absorbance was read at 405nm.

Blanks were prepared by adding the reagents in reverse order without incubation, and the values obtained subtracted from the test values.

A typical standard curve is shown in Fig 4. Activity was stable at -20°c for up to 3 months. Inter-assay coefficient of variation (CV) was 8.2% and intra-assay CV, 6.5%. Normal range in 25 healthy subjects was 108 $\pm$ 18% of the normal

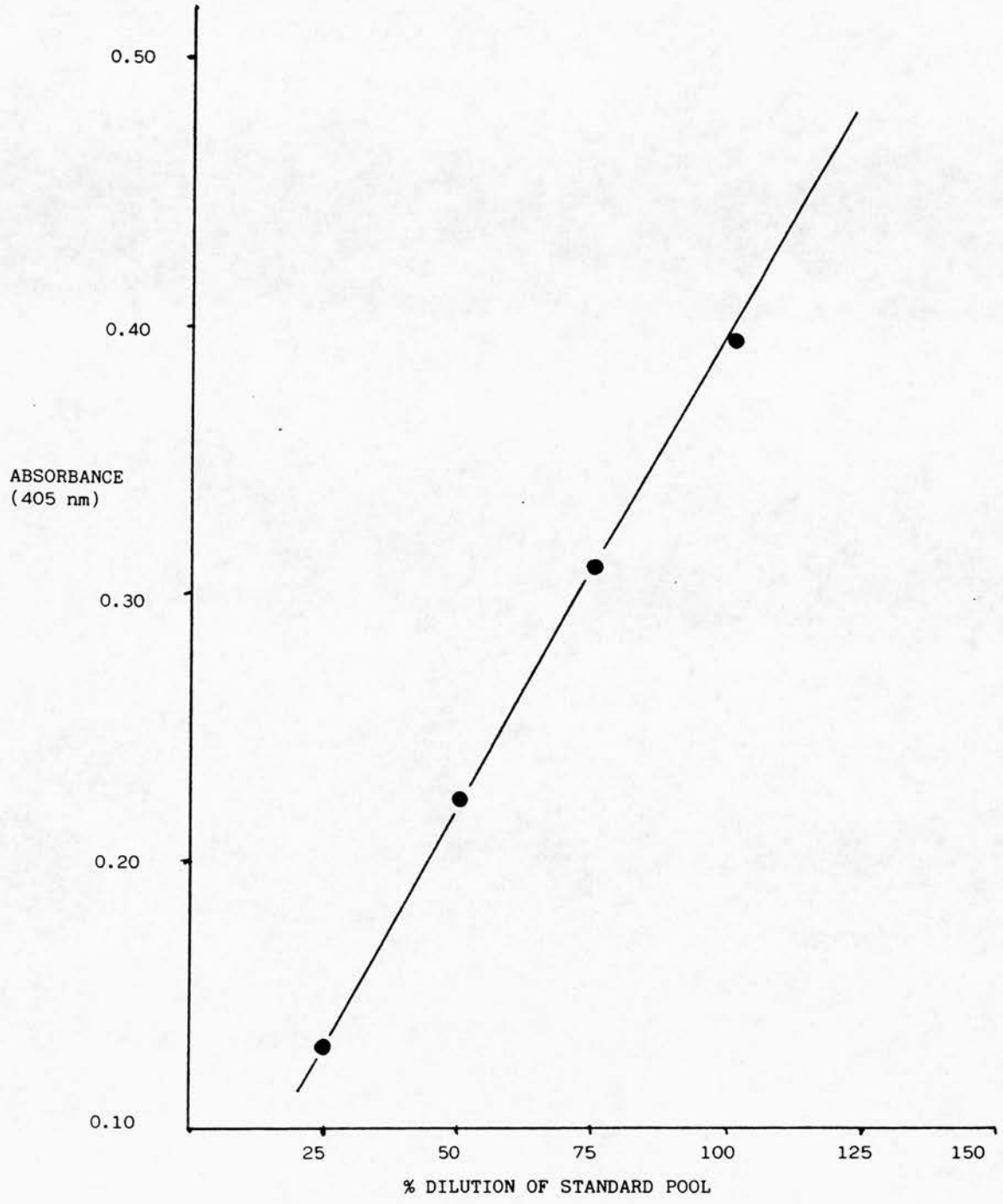


Figure 4. Plasma pre-kallikrein standard curve

pool (mean ± 2.5 SD). Gallimore et al have shown a correlation between prekallikrein concentrations measured by this assay and by an immunochemical method (rocket immunoelectrophoresis) of 0.84.

2.3.2. "Kallikrein-like" activity

This was measured as described by Gallimore and Friberger (1982), using an identical method to the prekallikrein assay, but substituting buffer for the prekallikrein activator. Test plasma was diluted as for the 100% standard. None of the normal plasmas generated an absorbance significantly different from the blank.

2.3.3. Urinary kallikrein

This was measured by the method of Amundsen et al (1979). This assay has recently been validated by comparison with measurement of urinary kininogenase activity using a kinin radioimmunoassay (Bonner and Marin-Grez, 1981). Urine for kallikrein estimations was taken directly into plastic containers, and frozen at -20°c after a maximum of 24 hours at room temperature. Samples were thawed at 20°c, and centrifuged prior to assay; all samples were assayed within 4 hours of thawing.

Urinary kallikrein was measured using the chromogenic substrate S-2266 (H-D-Val-Leu-Arg-pNA)(AB Kabi Diagnostica). A Tris buffer, pH 8.2, was prepared as above.

A Trasylol (lyophilised aprotonin, Bayer FRG) buffer was prepared to a concentration of 20 KIU/ml. This was added to a sample blank for each assay. Since Trasylol is a potent

inhibitor of urinary kallikrein, but does not inhibit urokinase, the only other alkaline esterase enzyme in urine, subtraction of the result of this blank from the sample value increased the specificity of the assay, in addition to correcting for the intrinsic urine colour.

Determination of urinary kallikrein

H-D-Val-Leu-Arg-pNA+H2O---urinary kallikrein--->peptide+pNA

500 µl of buffer at 37°c was added to 400 µl urine and incubated at 37°c for 5 minutes. 100 µl of S-2266 was added and incubated for 30 minutes. The reaction was then stopped by addition of 100 µl of 50% acetic acid. A sample blank was prepared, identical other than the use of the Trasylol buffer. The absorbance of each sample was read against its blank at 405 nm. Results were expressed as nkat/l, one nkat being the amount of glandular kallikrein which cleaves 0.05 umol of substrate per minute under the given conditions. This was calculated as nkat/l = 146 x absorbance.

Urinary kallikrein was stable at -20°c for up to 1 year; all samples were assayed within 3 months. Inter-assay CV was 8.2% and intra-assay CV 4.5%.

It has been suggested that removal of kallikrein inhibitors from the urine by dialysis or gel filtration is necessary when measuring urinary kallikrein activity, and that dilution of the sample may influence the result by altering kallikrein - inhibitor binding (Zschiedrich et al, 1980). We measured urinary kallikrein activity as above in 19 4-hour

urine collections from normal subjects; we measured neat urine, urine diluted 1:2 with assay buffer, and urine eluted from a column of Sephadex 150 (Sigma), 15cm in length and 2cm diameter. The eluate collected 3-6 minutes after application of the sample to the column contained virtually all the kallikrein activity. As shown in Table 3, neither gel filtration nor sample dilution had any significant effect on the urinary kallikrein activity. Similarly, dialysis for 24 hours against running tap water had no effect on kallikrein activity.

	Mean	SD	SEM
Urine volume (ml/4h)	311.4	165.7	38.0
Urine sodium (mmol/l)	140.6	61.8	14.2
Urinary kallikrein activity (nkat/l)			
Neat urine	11.21	6.60	1.51
Gel filtration	11.46	8.13	1.89
Dilution 1:2	11.85	7.07	1.62

Mean coefficient of variation between three assay methods, - 15.4%

Table 3. Analysis of 19 4-hour urine collections from normal subjects.

To investigate the influence of urine pH and glucose concentration on urine kallikrein activity, urine aliquots from 5 healthy subjects were titrated to pH5 with hydrochloric acid and to pH8 with sodium hydroxide. Glucose was added to a concentration of 1% and 2%. Results are shown in Table 4.

	Kallikrein concentration (nkat/l) (mean+SD)
Urine from 5 normal subjects	11.66 ± 7.20
pH 8	10.75 ± 6.53
pH 5	11.27 ± 7.14
1% glucose	11.98 ± 7.45
2% glucose	12.39 ± 7.41

Table 4 Effect of pH and glucose concentration on urine kallikrein activity.

The stability of urine kallikrein activity at room temperature was tested by placing freshly voided normal urine in a glass or a plastic container; 5 ml aliquots were taken at 30 mins, 1 hour, 4 hours, 8 hours, and 24 hours. Results are shown in Figures 5 and 6. There was no change in kallikrein activity with time; the mean coefficent of variation in plastic containers was 4.25% and in glass containers, 6.8%.

Normal range for 24 hour urinary kallikrein excretion in 20 normal volunteers was 11 ± 5.6 nkat/24 hours. There was no diference between male and female subjects (males 10.2 ± 4.1 nkat/24h, n = 11, female 12 ± 7.4 nkat/24h, $p > 0.05$). Studies in 10 normal subjects confirmed a diurnal variation in kallikrein excretion as previously described (Abe et al, 1981; Bell et al, 1987). A median value of 83% (range 73 - 91%) of the daily urinary kallikrein was excreted between rising in the morning and retiring at night, and 17% (range 9 - 27%) during nocturnal recumbency.

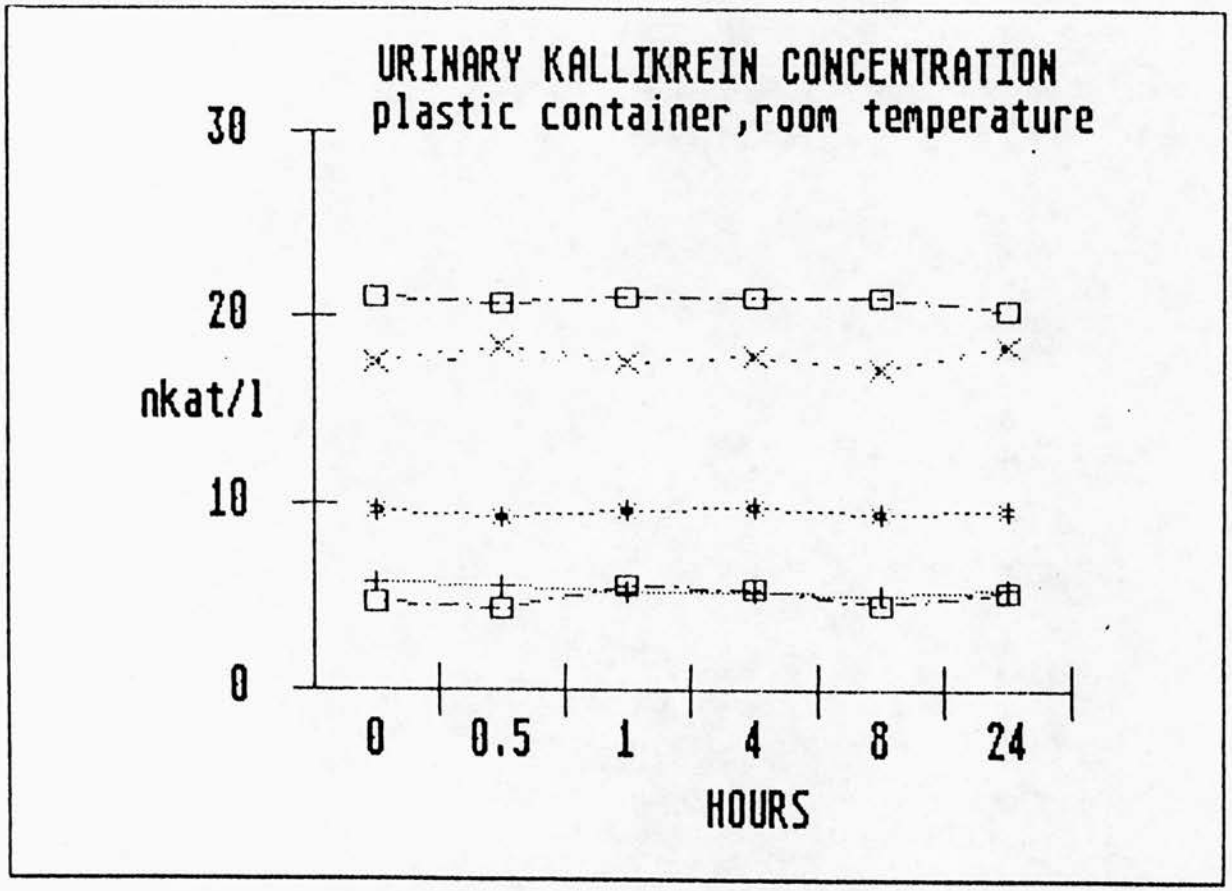


Figure 5.

Kallikrein concentration in urine from 5 healthy subjects, over 24 hours at room temperature in a plastic container.

Mean coefficient of variation 4.25%

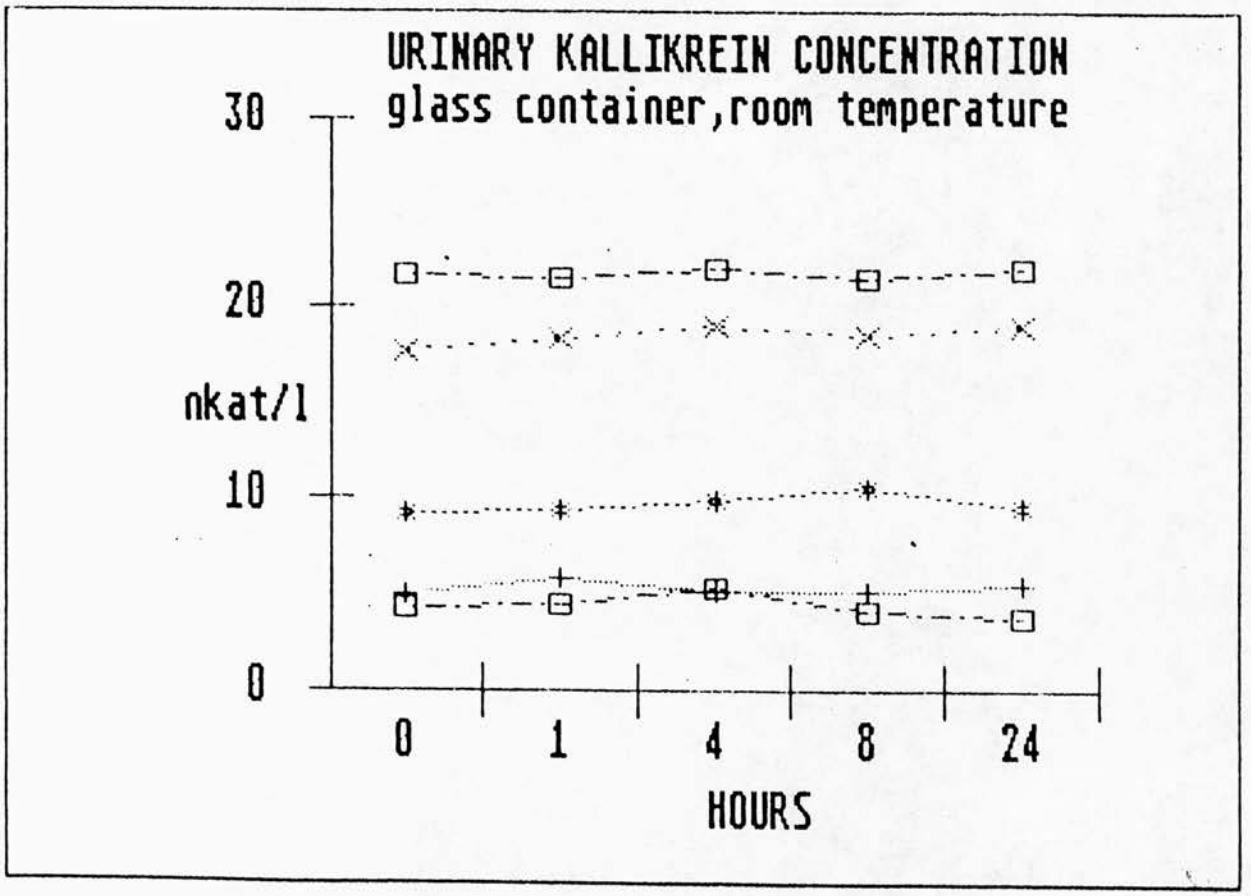


Figure 6.
Kallikrein concentration in urine from 5 healthy subjects, over 24 hours at room temperature, glass container.

Mean coefficient of variation 6.8%

Statistical analysis was performed using either the "Oxstat" or "Minitab" statistical packages. The standard tests employed were the Student's t-test and Mann-Whitney U test for comparison of group means; linear product-moment correlation coefficients for analysis of correlation, with log transformation of non-parametric data; and one-way analysis of variance to assess changes in a variable with time. Where ANOVAR showed such a change, mean values at each time-point were compared with baseline values by Student's t-test. Unless otherwise stated, results are shown as mean $\pm$ SD, and values of $p < 0.05$ were taken as significant. I am grateful to Dr Robert Elton (Dept of Medical Statistics, University of Edinburgh) for statistical advice in relation to several parts of this work.

CHAPTER 3 URINARY KALLIKREIN EXCRETION DURING SALINE INFUSION IN NORMAL MAN

3.1. If the renal kallikrein-kinin system is involved in the regulation of sodium excretion, then the rapid administration of a large saline load would be predicted to influence urinary kallikrein excretion, and some correlation would be expected between kallikrein excretion and sodium excretion. This hypothesis was tested by infusion of 3 litres of normal saline over 1 hour into healthy volunteers.

3.2. Subjects and methods

Ten normal male subjects (age 30 ± 6 years) with no history of renal disease or hypertension were studied.

Protocol

Subjects were fasted from 22.00 hours on the evening before the study, and were asked not to smoke. At 08.00 the following morning catheters were inserted into a vein in each ante-cubital fossa. A control blood sample was withdrawn. An infusion of 5% dextrose containing 25 g/l inulin and 1.15 mmol/l para-aminohippurate (PAH), for measurement of glomerular filtration rate and renal plasma flow respectively, was commenced at rate of 1 ml/min and continued throughout the study. Sodium chloride solution 150 mmol/l was infused at 50 ml/min between 10.00 and 11.00 hours. Timed urine collections of approximately 30 minutes each were made between 09.00 and 13.00 hours, apart from during the saline infusion when urine was collected at 20 minute intervals. Subjects stood to void urine but otherwise

remained recumbent. Blood samples were withdrawn every 30 minutes. Blood pressure was measured with a standard sphygmomanometer.

Urinary kallikrein excretion was measured as above. Plasma renin activity (PRA) was measured by RIA of generated Angiotensin I, using an established method which has been fully validated in our laboratory (Roulston et al, 1983). Plasma and urinary PAH and inulin were measured by autoanalyser techniques (Harvey and Brothers, 1962; Dawborn, 1965); plasma and urinary sodium and plasma protein concentration were measured by standard laboratory methods.

The 20 minute urine collections during saline infusion, the 30 minute collection immediately after the infusion, and the final 30 minute collection (150-180 minutes after the start of the infusion) were analysed for kallikrein and the results included in statistical analysis. Results are expressed as mean $\pm$ SEM. Results for each variable were compared by analysis of variance, and if a significant trend was observed, results for each clearance period were compared by Student's t-test. Standard correlation coefficients were calculated. Values for $p < 0.05$ were taken as significant.

3.3. Results

There were no significant changes in systemic blood pressure during the study. Urinary sodium excretion increased significantly during saline infusion and remained elevated at 90 and 180 minutes (Fig 7). There was a significant

increase in urine flow (Vu) during saline infusion which persisted at 90 minutes (Fig 7). Vu subsequently fell and was only slightly above control values at 180 minutes. In 8 out of 10 subjects, renal plasma flow increased during and after the infusion, returning to control values at 180 minutes; these trends were not statistically significant because of considerable variability among subjects (Fig 8). GFR showed a similar pattern, and again the changes did not achieve statistical significance (Fig 8). PRA decreased significantly after saline infusion ($p<0.01$) and remained low at 180 minutes (Fig 9). Urinary kallikrein excretion increased rapidly during the saline infusion, with peak activity in the 40-60 minute collection, persistent elevation at 90 minutes, and a return to control values at 180 minutes (Fig 9). Plasma protein concentration decreased at 90 minutes (control 63.8 $\pm$ 1.7 g/l, 90 min 52.5 $\pm$ 2.4 g/l, $p<0.01$) and was still depressed at 180 minutes (56.3 $\pm$ 0.8 g/l, $p<0.01$).

Over the whole study there were weak correlations between urinary kallikrein excretion and urine flow (r 0.45, $p<0.01$) and urinary sodium excretion (r 0.35, $p<0.02$). These correlations may have achieved significance because of multiple observations in each individual.

In order to further assess the relationships between PRA, kallikrein , urine flow and urinary sodium, studies of correlation were performed on the results of blood and urine samples taken in the control period prior to saline infusion (A), between 60 and 90 minutes into the study (immediately

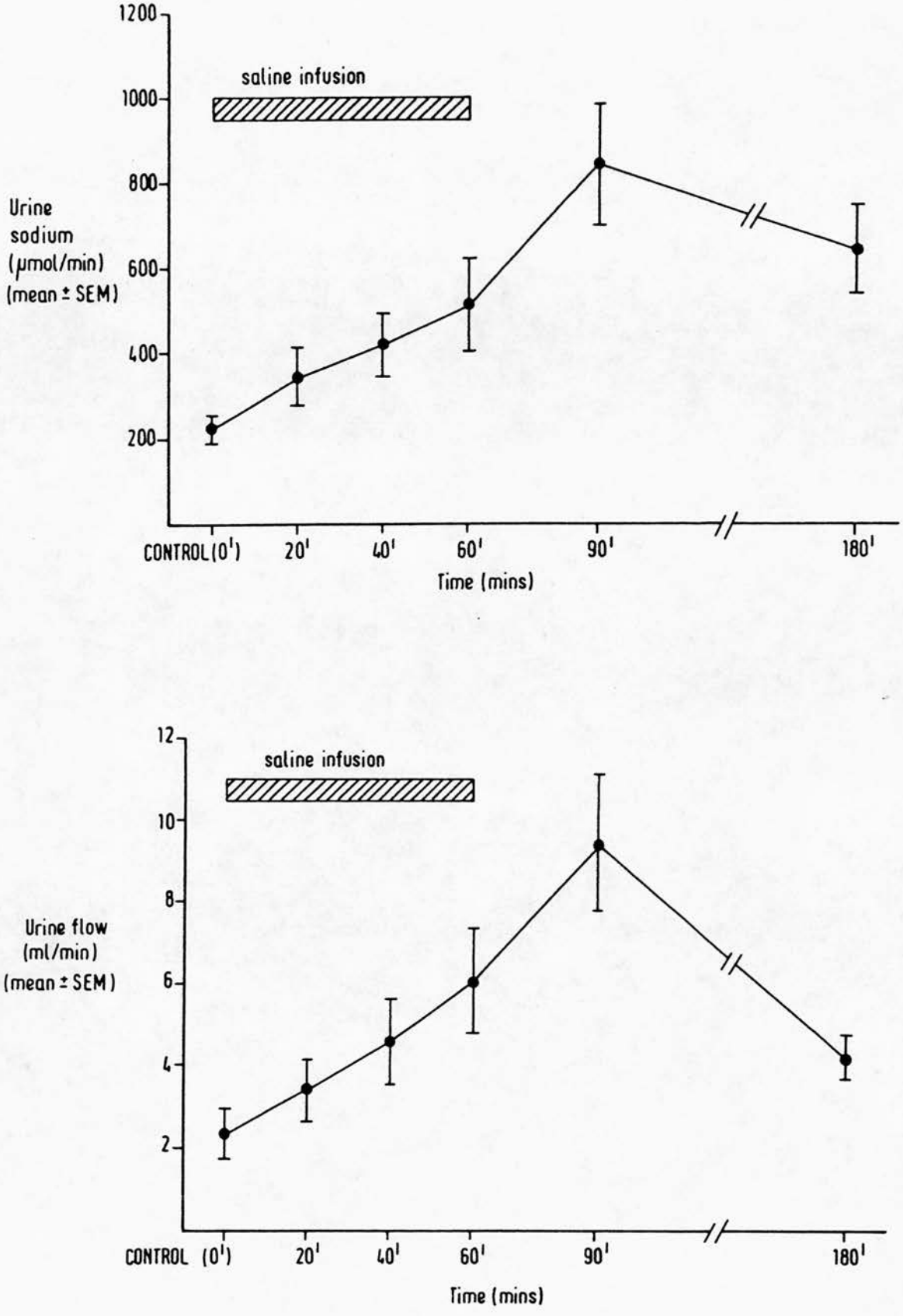


Figure 7. Urine sodium excretion and urine volume, saline infusion study

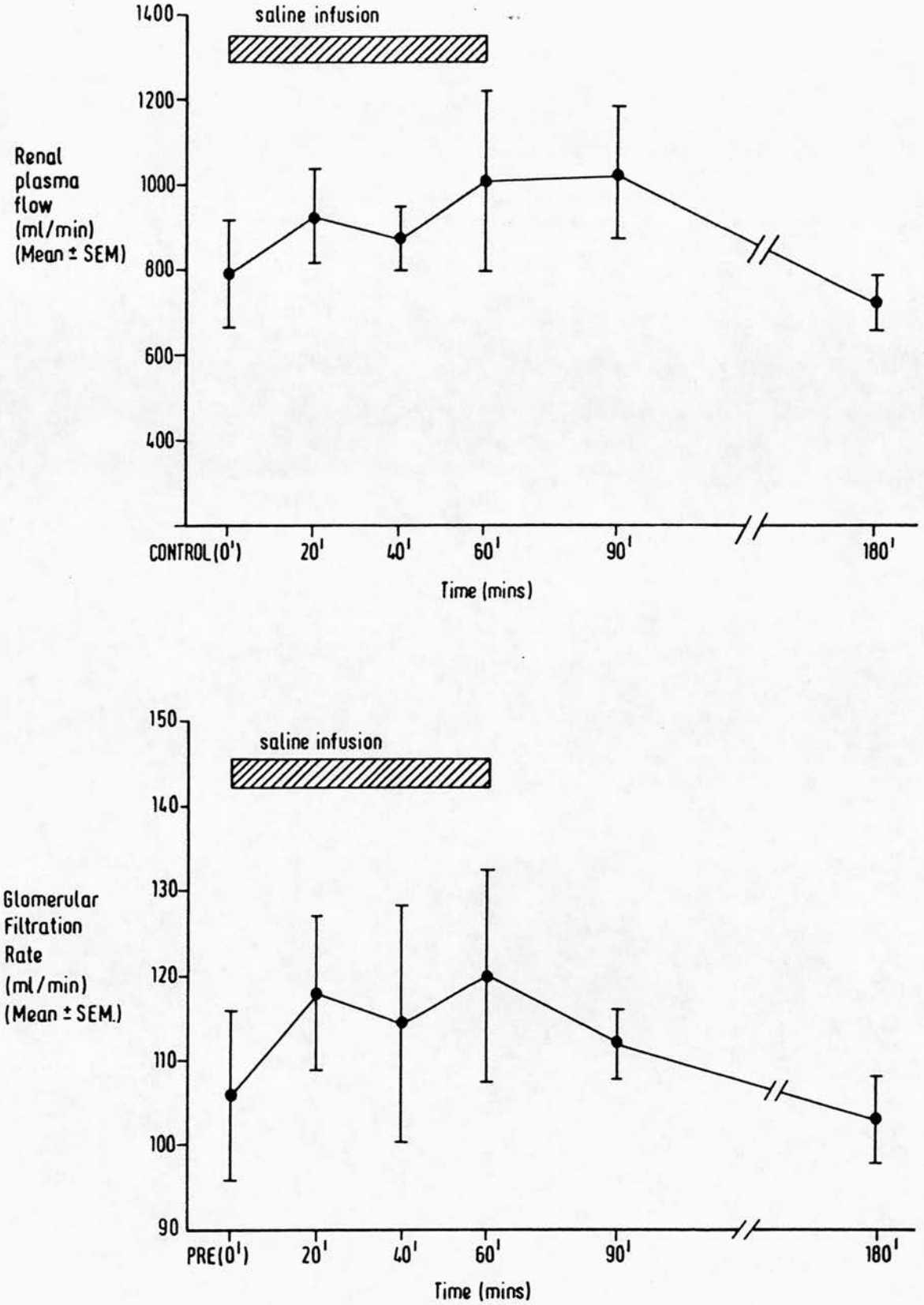


Figure 8. PAH and inulin clearance, saline infusion study.

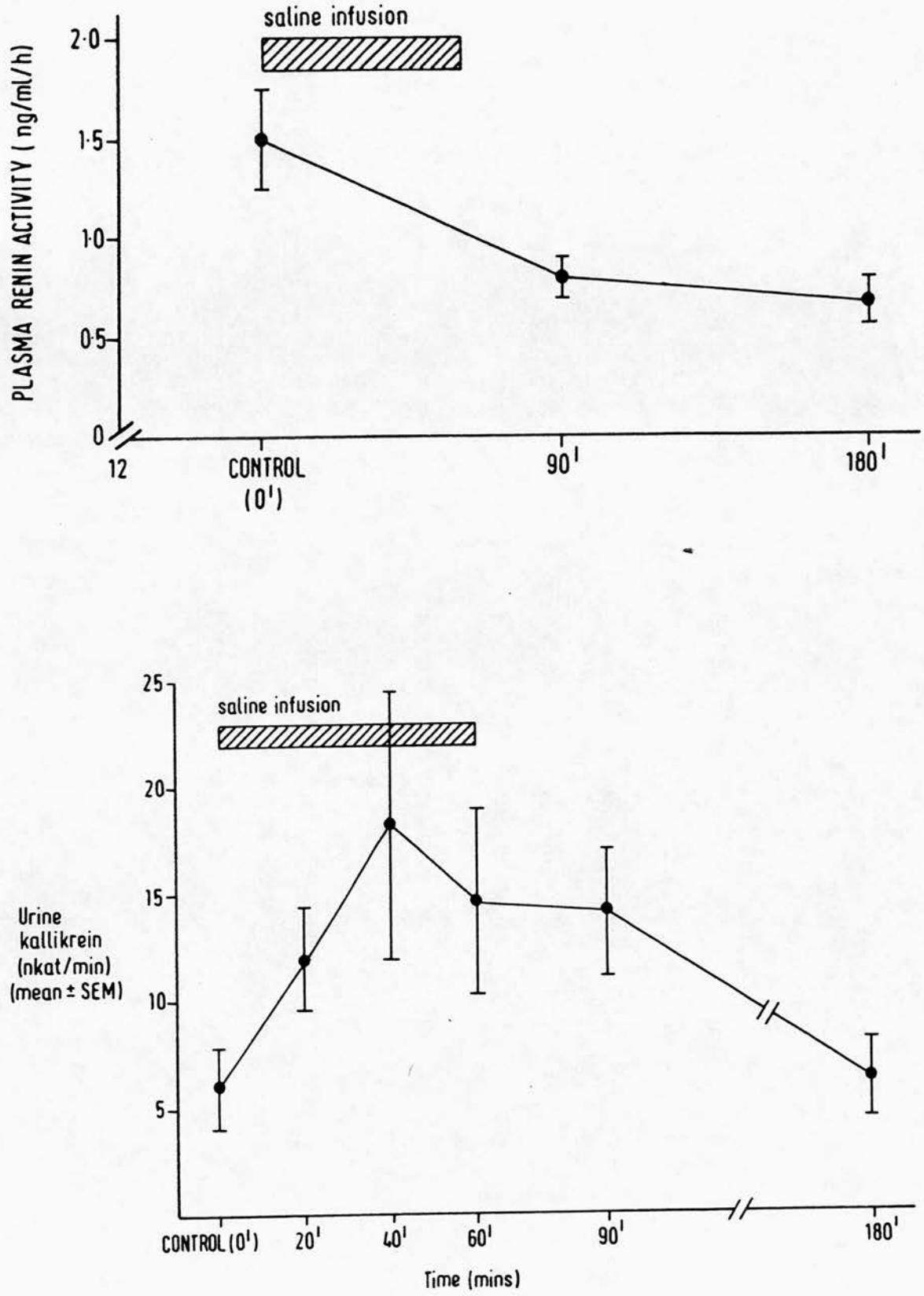


Figure 9. PRA and urinary kallikrein excretion, saline infusion study

after the end of the infusion) (B), and between 150 and 180 minutes into the study (C). The correlations obtained by including all these observations are shown in Table 5. Both PRA and urinary kallikrein excretion correlated significantly with Vu and UNaV; the correlation coefficient (r) with UNaV was slightly higher than with Vu in each case. There was also a significant inverse correlation between PRA and urinary kallikrein excretion, suggesting a reciprocal linkage between these two systems. To assess the possible importance of this phenomenon, a ratio of PRA to UKallV was calculated. The PRA/UKallV ratio fell after saline infusion and was still depressed at 180 minutes (Fig. 10) The ratio showed a non-parametric distribution and was therefore log-transformed for the purposes of correlation analysis. There was an extremely high degree of correlation between this ratio and UNaV (r 0.868), and Vu (r 0.811).

	PRA	UKallV	Vu	UNaV	log PRA/UKallV
PRA	-	-0.58**	-0.66**	-0.74**	-
UKallV	-0.58**	-	0.75**	0.77**	-
Vu	-0.66**	0.75**	-	-	-0.81***
UNaV	-0.74**	0.77**	-	-	-0.87***
log PRA/ UKallV	-	-	-0.81***	-0.87***	-

** p<0.01
*** p<0.001

Table 1 Correlations between variables in saline infusion study (periods A, B, and C).

These correlations might have been due in part to similar directional changes in each variable in each subject. To

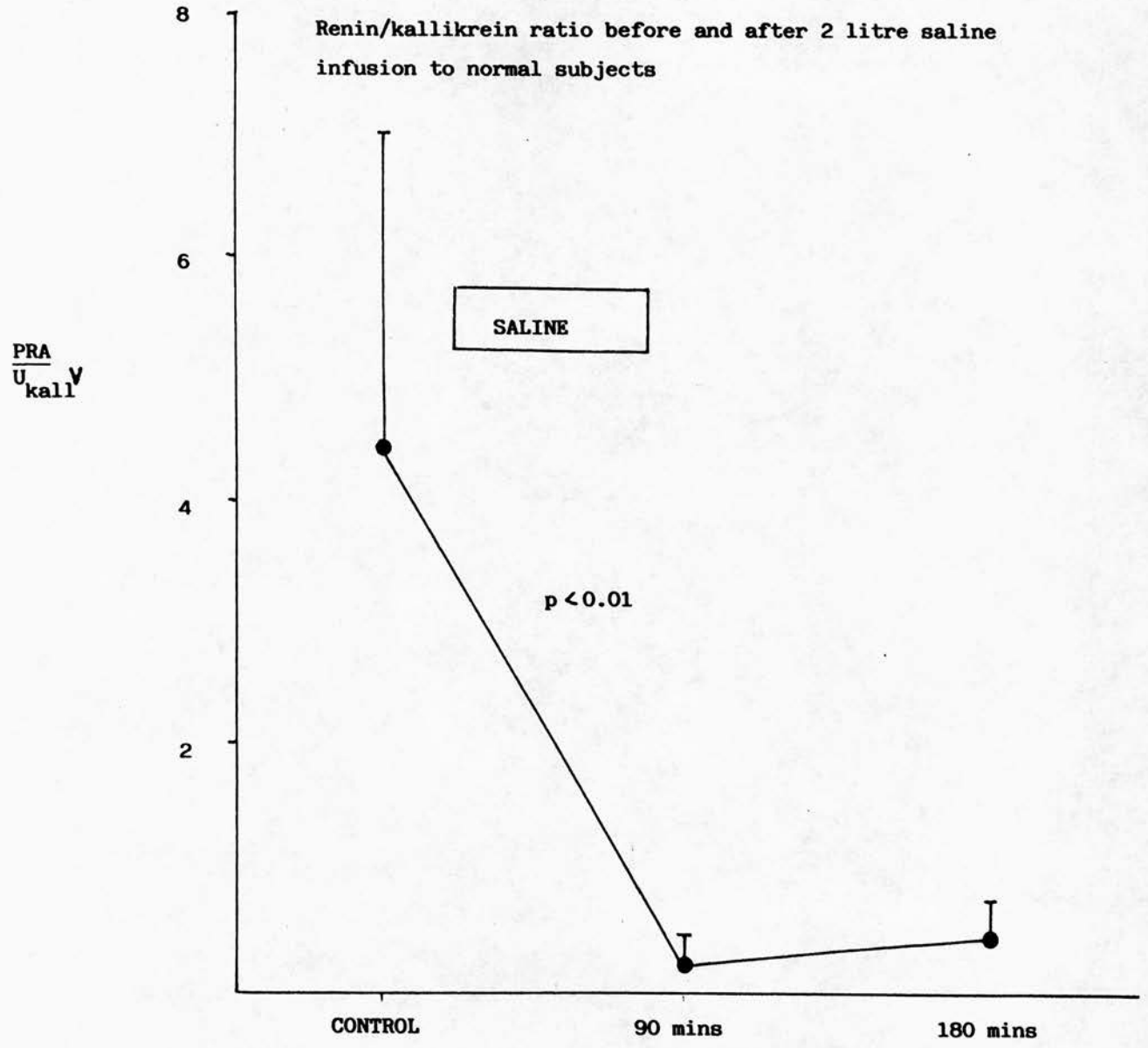


Figure 10. Ratio of PRA to UKallV, plotted against time ; saline infusion study.

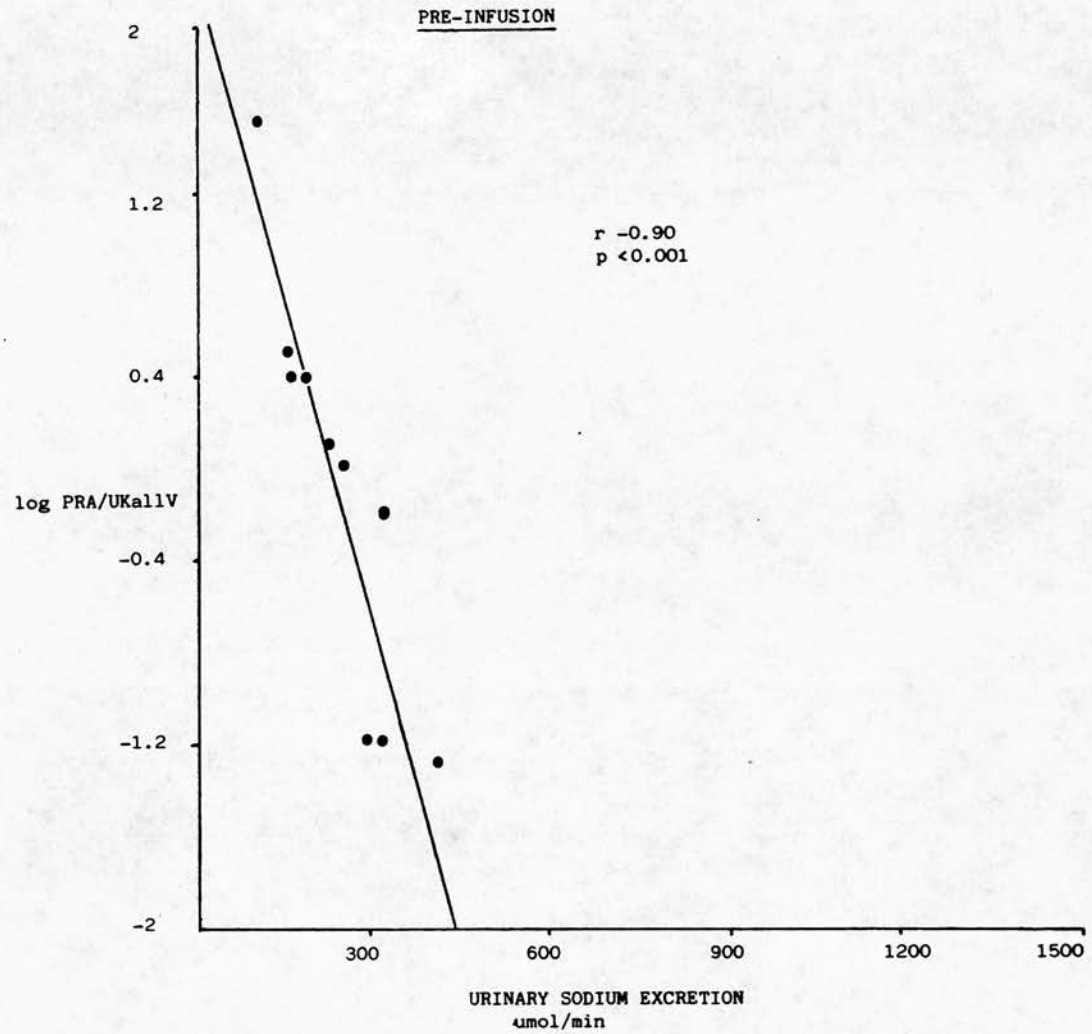


Figure 11. Log PRA/UKallV aginst UNav prior to saline infusion.

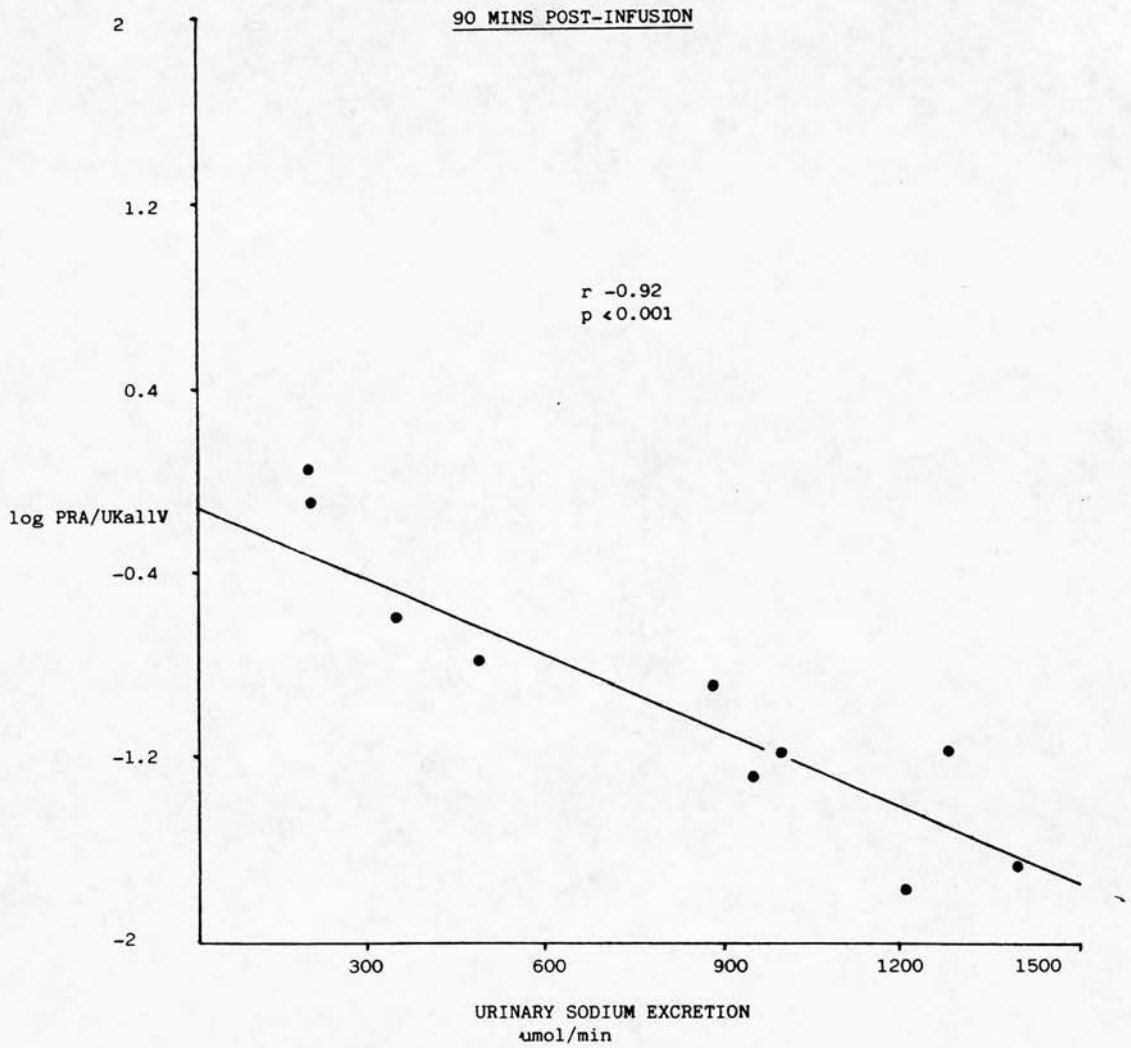


Figure 12. Log PRA/UKallV against UNaV, 90 minutes after start of infusion.

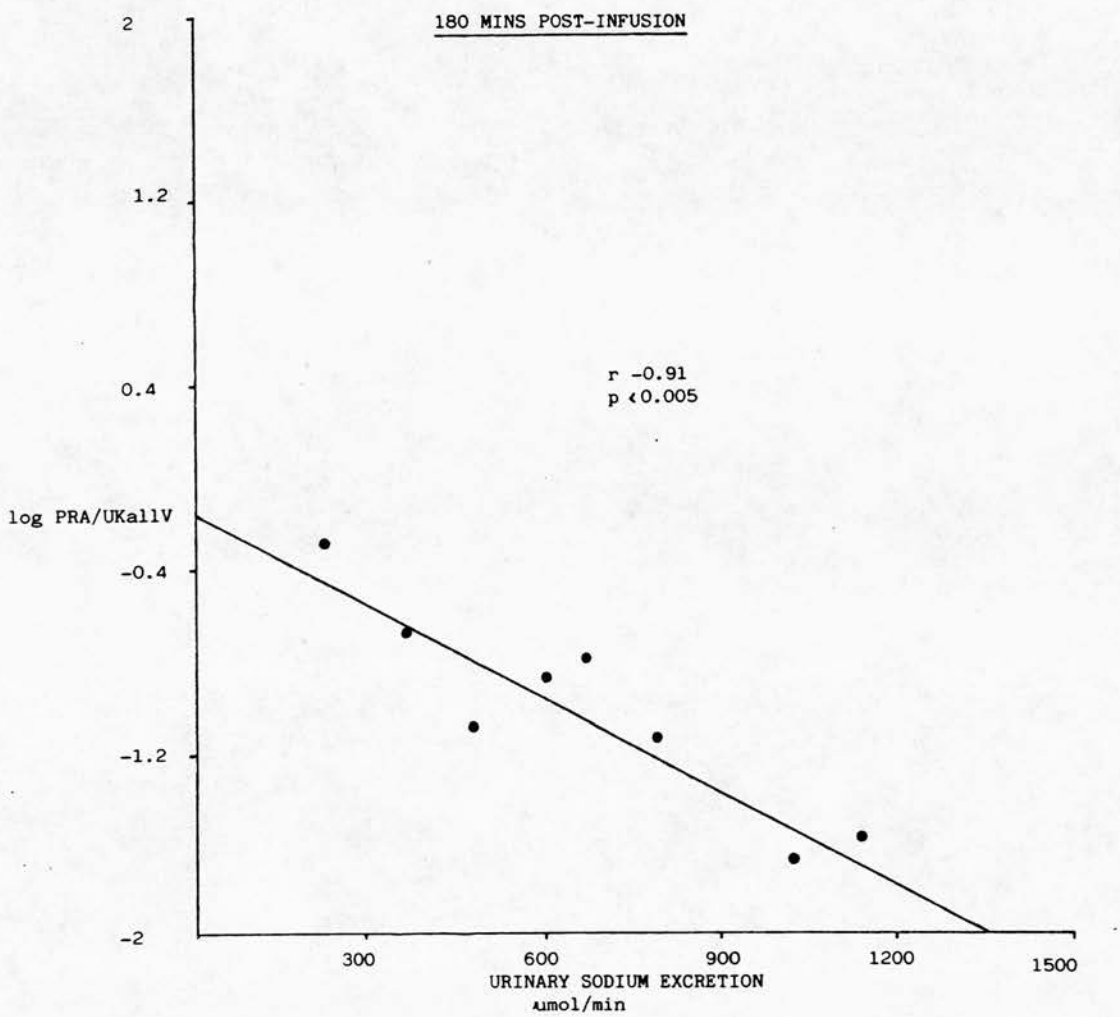


Figure 13. Log PRA/UKallV against UNaV, 180 minutes after start of infusion.

2 subjects unable to provide appropriate sample

test this, similar correlations were calculated for each of the 3 individual study periods in the 10 subjects. The correlation coefficients between log PRA/UKallV and UNaV were in fact increased when individual clearance periods were considered, and were 0.90 or greater in each case. As shown in Figs 11, 12, and 13, the slope of the regression line between log PRA/UKallV and UNaV differed markedly in each clearance period. The pattern of slopes indicated that in the control period, changes in log PRA/UKallV were associated with relatively small changes in UNaV; after saline infusion, the increased steepness of the regression lines indicated that changes in log PRA/UKallV were associated with much greater changes in UNaV.

3.3. Discussion

In previous studies, some workers have failed to demonstrate an increase in kallikrein excretion after acute volume expansion, and have not observed any correlation with sodium or water excretion (Levy, Frigon and Stone, 1978; Marks and Keiser, 1983; Lewis et al, 1988). However, Mills and colleagues found that infusion of either saline or 2.5% dextrose increased kallikrein excretion in the dog; kallikrein excretion correlated with urinary sodium excretion and urine flow after saline infusion (Mills, 1976). In the present study, saline infusion in man produced a prompt and significant increase in kallikrein excretion, which preceded the maximal increase in urine volume and sodium excretion. This conforms to the predicted response if

the renal kallikrein-kinin system has a role in the early diuretic and natriuretic response to acute volume expansion. Support for this concept comes from studies showing that administration of anti-bradykinin antibodies , or of the kallikrein inhibitor aprotonin, reduce sodium and water excretion after saline infusion (Marin-Grez, 1974; Kramer et al, 1984). It is difficult to reconcile these results with the hypothesis that the renal kallikrein-kinin system is a sodium retaining mechanism. While there was a correlation with urine flow rate, this was weaker than the correlation with urinary sodium excretion; the opposite would be expected if passive "wash-out" was the major factor controlling UKallV.

In this study, urinary kallikrein excretion and PRA changed in an inverse fashion during saline infusion, and there was a statistically significant inverse correlation between them. Such reciprocal changes in putative markers of activity of the renal kallikrein-kinin system and the renin-angiotensin system, could have considerable significance. The biological consequences of activation of these systems, at least in terms of renal function, are almost certainly opposite, with the renin-angiotensin system mediating renal vasoconstriction and sodium retention, and the kallikrein-kinin system favouring vasodilation and natriuresis, as outlined above (Chapter 1, p12; Mills, 1972). It follows that the observed increase in kallikrein excretion and decline in PRA would favour renal vasodilation and natriuresis to a greater extent than either change in

isolation. In keeping with this hypothesis is the observation that an "activity ratio" between the renin-angiotensin system and the renal kallikrein-kinin system, obtained by dividing PRA by UKallV, as described by Levy et al (1977), was a very strong statistical determinant of sodium excretion in this study, with an r value in the region of 0.9. This indicates that approximately 80% of the variability in sodium excretion in this study may be attributed to changes in this ratio. Levy et al found a correlation between this ratio and renal blood flow in patients with hypertension; this correlation was absent in our study. To my knowledge the association with sodium excretion has not been previously documented.

It is of course apparent that other factors are involved in the early response to saline infusion. Interestingly, plasma atrial natriuretic peptide (ANP) and UKallV show a very similar pattern of response, with an early rise during volume expansion, followed by a fall to baseline values (Lewis et al, 1988). It has been suggested that the primary role of ANP is to promote renal vasodilation during volume expansion; evidence for a direct tubular effect is less convincing (Raine, Firth and Ledingham, 1989). ANP infusion at high dosage increases UKallV in rats by up to 80% (Thibault et al, 1984; Brownlee et al, 1986); while ANP has not been shown to increase urinary kallikrein excretion in man, it suppresses PRA (Weidmann et al, 1986), and would therefore be expected to decrease the PRA/UKA ratio. Recent

studies have indicated that the natriuretic effect of a given plasma concentration of ANP is very much greater if it is induced by saline infusion, than if it is produced by infusion of exogenous ANP. Other factors, eg renal dopamine, renal vasodilator prostaglandins, and suppression or inhibition of ADH, are also probably involved in the renal response to acute volume expansion (Ball and Lee, 1977; Bennet, Tighe and Wegg, 1982; Abe et al, 1981; Yamada et al, 1989), and the fall in plasma protein concentration should theoretically promote natriuresis (Green, Windhager and Geibisch, 1974). Such mechanisms could be relevant to the increased slope of the regression line between the PRA/UKAllV ratio and sodium excretion after volume expansion, which is in keeping with a degree of "gain" influencing this relationship. This possibility will be discussed further in the final chapter.

CHAPTER 4 ASSOCIATIONS BETWEEN PLASMA RENIN ACTIVITY, URINARY KALLIKREIN EXCRETION, URINE VOLUME AND URINARY SODIUM EXCRETION UNDER BASAL CONDITIONS IN NORMAL MAN

4.1. Introduction

As documented in Chapter 3, a significant correlation was observed in man between the ratio of PRA to urinary kallikrein excretion (log PRA/UKallV) and urinary sodium and water excretion. This correlation held both after and, surprisingly, before acute volume expansion by saline infusion. This suggested that reciprocal changes in the activity of the renin-angiotensin and kallikrein-kinin systems in the kidney might have a regulatory influence on renal function even under basal, unstimulated conditions. To test this hypothesis further, these variables were measured in normal subjects at rest, over a 9 hour period, under conditions of modest water diuresis.

4.2. Subjects and methods

11 healthy male volunteers, with no history of renal disease or hypertension and on no medication, were studied. After an overnight fast, infusions of PAH and inulin were established as in Chapter 3. Subjects drank 100 ml water at the start of the study and hourly thereafter. Blood and urine were sampled hourly for 9 hours. Subjects remained supine at rest during the study, other than rising to pass urine immediately after each blood sample. No drugs or other agents were administered during the study.

Variables measured were urine volume (Vu), urinary sodium excretion rate (UNaV), clearance of PAH (CPAH) and inulin (Cinulin), plasma renin activity (PRA) and urinary kallikrein excretion rate (UKallV). Methods were as previously described.

4.3. Results

Urine volume, Cinulin and CPAH remained constant during the study. There was a tendency during the study for urinary sodium excretion to fall, although this was not significant by one-way analysis of variance (Fig 14). This may have reflected the absence of sodium intake during the study. There were also non-significant trends for UKallV to fall and PRA to rise (Fig 15). There was a weak but significant correlation between PRA and Vu (r -0.26, $p<0.01$). The correlation between PRA and UNaV was relatively strong (r -0.48, $p<0.001$). UKallV did not correlate significantly with Vu, but correlated weakly with UNaV (r 0.27, $p<0.01$). The ratio of PRA to UKallV (log PRA/UKallV) did not correlate with Vu (r -0.20) but correlated very strongly with UNaV (r -0.51, $p<0.0001$). There was no significant correlation between PRA and UKallV in this study (r 0.10, $p>0.05$).

4.4. Discussion

The subjects in this study, while water replete throughout, showed some evidence of a renal response to ongoing negative sodium balance over the 9 hour period. These changes were not however of sufficient magnitude or consistency to

achieve statistical significance. Studies of correlation suggested an influence of both renin and kallikrein on sodium excretion under these conditions, with renin as perhaps the stronger determinant. When these variables were combined as a ratio, a very highly significant relationship was observed with sodium excretion. One must of course interpret the results of correlation studies with great caution; in particular, causality cannot be assumed on such a basis. Nevertheless the findings are again in keeping with a role of renin-kallikrein interactions in the regulation of sodium excretion, and suggest that this relationship may be important under basal, unstimulated conditions in man.

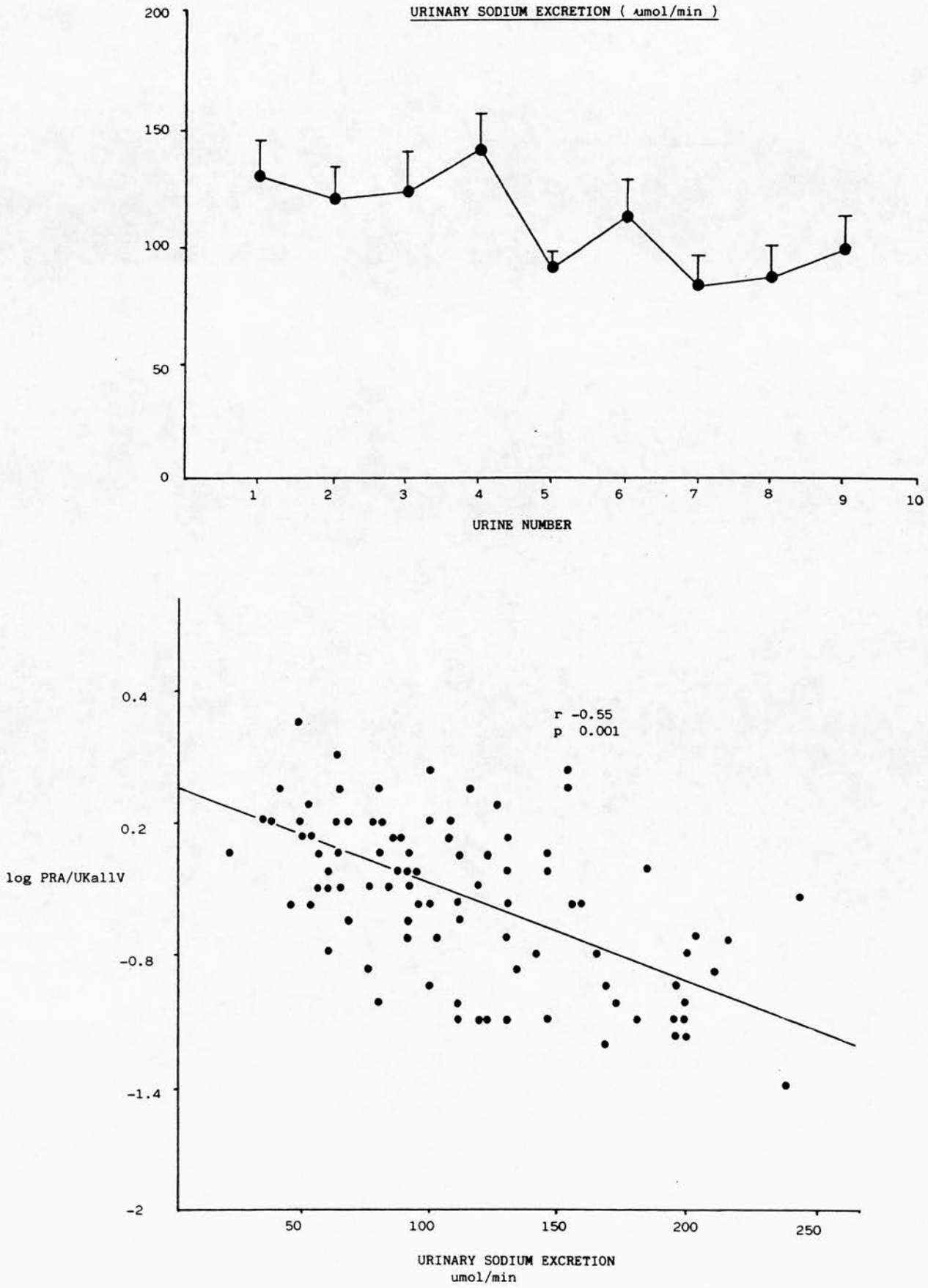
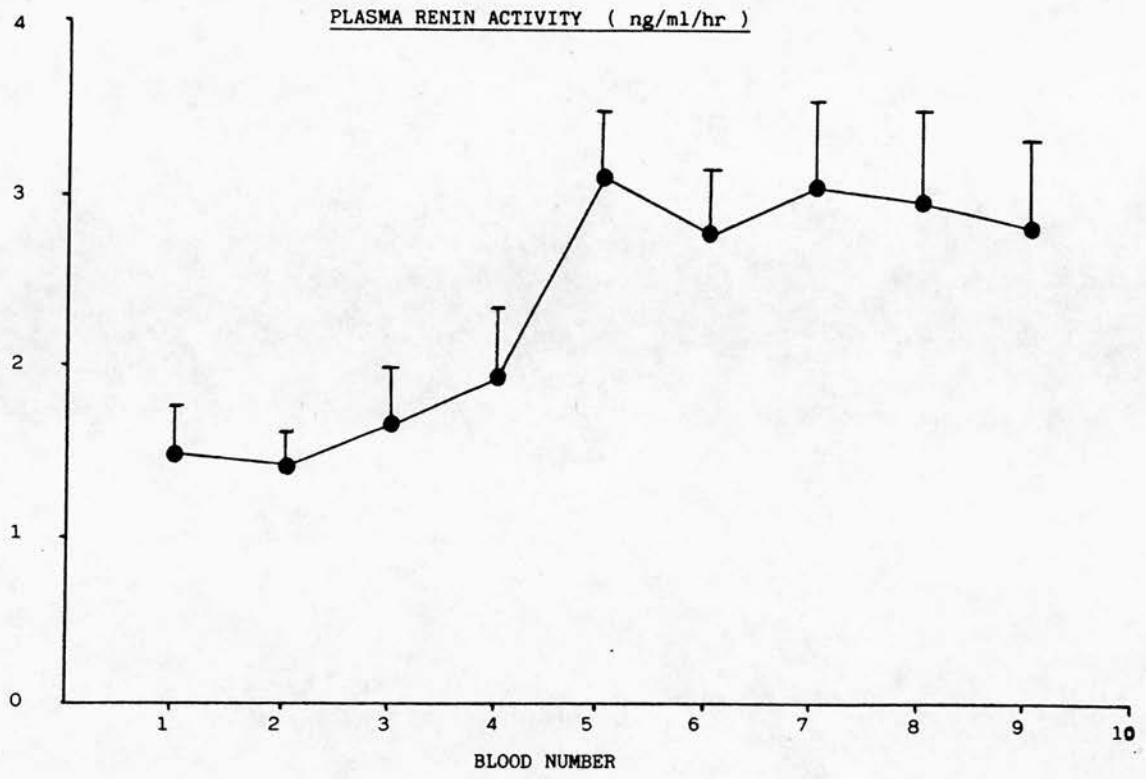


Figure 14. UNaV during "basal" study, and overall correlation of log PRA/UKallV with UNaV.



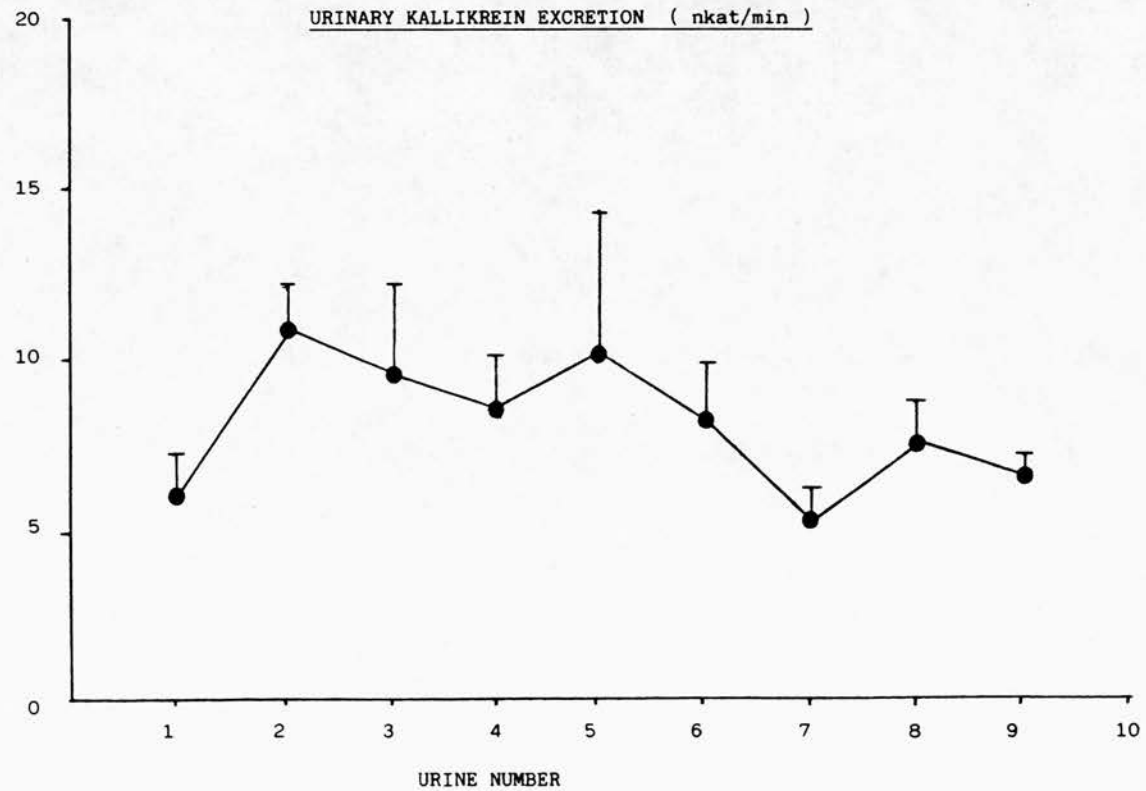


Figure 15. PRA and UKallV, "basal" study.

CHAPTER 5 CELLULAR DISTRIBUTION OF RENAL KALLIKREIN; THE GLOMERULAR PERIPOLAR CELL

5.1. Introduction

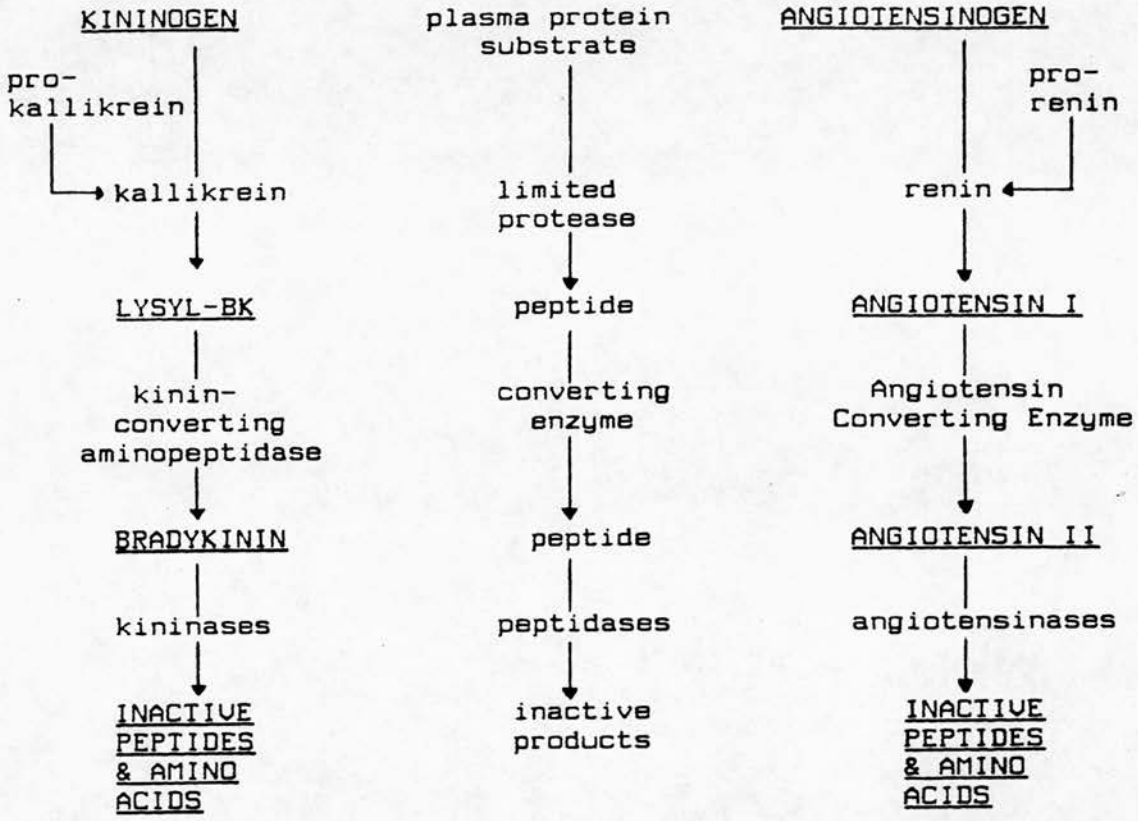
The results described in Chapters 3 and 4 appeared to indicate interesting relationships between the renal kallikrein-kinin system, the renin-angiotensin system, and renal function. As outlined in the introduction, there are other reasons to speculate that these two systems might operate in a counter-regulatory fashion. Attention has also been drawn to the very close homology between them in their enzymatic organisation, suggesting perhaps a parallel development in evolution (Fig 16)(Pisano, 1975). A very major objection to this hypothesis, however, is the apparent absence of any close anatomical relationship between renin- and kallikrein-containing structures in the kidney. This has been confirmed in a number of studies, which have found kallikrein to be confined to the cells of the distal convoluted tubule (Scicli et al, 1976; Orstavvik et al, 1976; Orstavvik and Inagami, 1982; Vio and Figueroa, 1985; Barajas et al, 1986). Orstavvik et al in 1982 described the results of an immunocytochemical study as follows; " Since the present study demonstrates the localisation of kallikrein at a tubular level distal to the juxtaglomerular apparatus, kallikrein released into the tubular lumen will not pass renin-containing structures. Moreover, at no point was a close anatomical relationship observed between the kallikrein- and the renin-containing structures. From an

anatomical point of view, it is therefore difficult to visualise how it would be possible for kallikrein within the kidney, either through the tubular lumen or the interstitial space, to reach and directly act upon renin-containing structures." Similar findings were reported by Vio et al in 1985. In 1979, Ryan et al first described the granular glomerular peripolar cell (PPC) in sheep (Ryan, Coghlan and Scoggins, 1979). These cells, thought to be of epithelial origin, lie in a "cuff" around the vascular pole of the glomerulus (Fig 17). They vary in number and prominence between species, being particularly obvious in sheep; one recent textbook stated that they do not exist in man, but careful studies have confirmed their existence in human kidneys (Gardner and Lindop, 1985). Exocytosis of granule contents into Bowman's space has been shown (Ryan et al, 1982), but the function of PPCs, and the nature of their secretory product, remains uncertain, although it is known that the cells do not contain renin (Gardner and Lindop, 1985). We therefore investigated the possibility that PPCs might contain renal kallikrein, using a specific antibody to human urinary kallikrein and immunoperoxidase staining.

5.3. Materials and methods

5.3.1. Tissue processing

Kidney tissue was obtained from normal sheep killed in an abbatoir; from the macroscopically normal parts of ten human kidneys removed by nephrectomy for carcinoma (7), calculus (2) and renal artery stenosis (1); and from needle biopsies



Pisano JJ ; Chemistry and biology of the kallikrein-kinin system. 1975.

Figure 16. Homology between the kallikrein-kinin system and renin-angiotensin system.

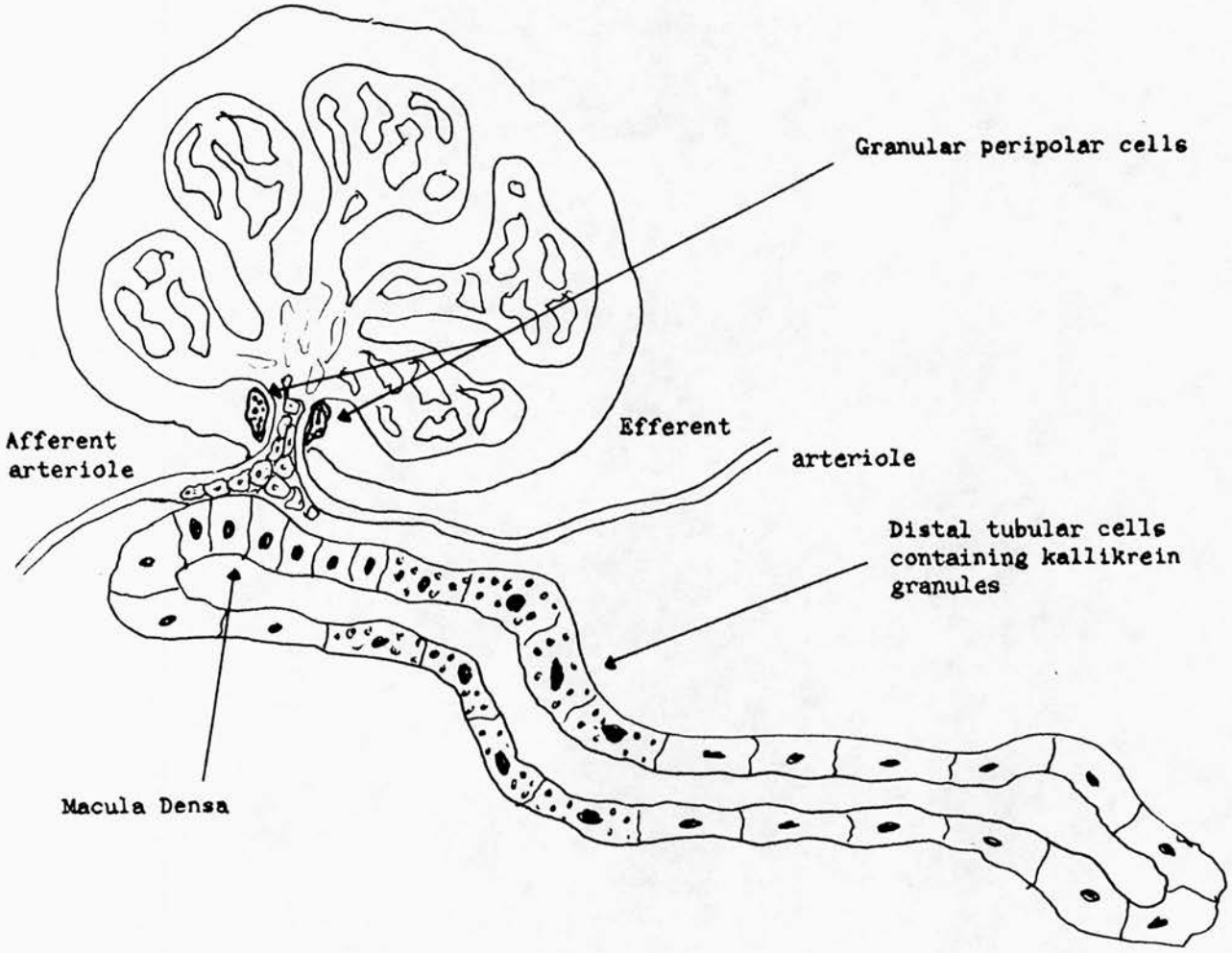


Figure 17. Location of glomerular peripolar cells.

of patients with renal disease. For light microscopy, kidney tissue was fixed in 10% formal saline and impregnated with paraffin wax at 56°C; sections were cut with a Lietz rotary microtome at a thickness of 3-4µm. For electron microscopy, tissue was fixed in glutaraldehyde, post-fixed in 1% osmium tetroxide, and embedded in araldite. Sections 1µm thick were cut and stained with toluidine blue. If PPCs were detected by light microscopy, 50-60nm sections were cut on an LKB III ultramicrotome, stained with uranyl acetate and lead citrate, and viewed on a Jeol 100S electron microscope.

5.2.2. Staining

Paraffin-embedded sections were stained by the Dunn's Crystal Scarlet method. This trichrome stain, in which Orange G, Brilliant Crystal Scarlet and Analine Blue are used sequentially, was found to be superior to other methods for the identification of PPCs in both sheep and human kidneys. In the process of the study, some sections were also stained with a toluidine blue/ Jones methenamine silver stain.

5.2.3. Immunocytochemistry

A highly specific polyclonal rabbit anti-human urinary kallikrein antibody was gifted by Dr Ervin Erdos, Dept of Pharmacology, University of Chicago, USA. The antibody was prepared by separating human urinary active kallikrein on DEAE-cellulose and octyl-Sepharose columns, and purifying it to homogeneity by affinity chromatography, gel filtration and hydrophobic h.p.l.c. An antibody to the purified preparation was raised in rabbits. The antibody was shown to

react with both active kallikrein and with prokallikrein, but not with plasma kallikrein or other protease enzymes (Takada, Skidgel and Erdos, 1985). The antibody was applied to paraffin-embedded sections of human kidney in concentrations from 1:20 dilution to 1:1000, and incubated correspondingly from 30 mins to 18 hours. Subsequent incubations were a) swine antirabbit antibody (Dakopatts, Glostrup, Denmark) at 1:30 dilution for 30 minutes b) rabbit peroxidase-antiperoxidase reagents (Dakopatts, Glostrup, Denmark) at 1:100 dilution for 30 minutes. Endogenous peroxidase activity was not observed. Control studies were carried out by preabsorbing the antibody with purified human urinary kallikrein (Protogen AG, Laufelfingen, Switzerland, supplied by Dr Michael Gallimore, Deal, UK).

5.3. Results

Light microscopy confirmed the location of PPCs at the vascular pole at the point of reflection of Bowman's capsule, and their close association with the afferent arteriole, but not the efferent arteriole (Fig 18). PPC cytoplasm was densely packed with granules in both sheep and human kidneys; human PPCs were fewer, less prominent, and had fewer granules than sheep cells (Fig 19). In both sheep and human tissue, PPCs were more numerous in the outer than the inner cortex.

Electron microscopy showed PPCs closely adherent to the basement membrane of Bowman's capsule (Fig 20). PPC granules

were round and homogeneous, but of variable diameter (200-1700 nm, mean 950 nm). Apparent exocytosis of granule contents into the urinary space was observed (Fig 21). PPCs had sparse rough endoplasmic reticulum and few mitochondria, suggesting a relatively low synthetic capacity. Renin granules were seen in myoepitheloid cells; they were sparse and smaller than PPC granules (mean diameter 350 nm). PPCs and myoepitheloid cells were often separated only by the basement membrane of Bowman's capsule (Fig 22).

The character and distribution of PPCs in renal biopsy tissue from patients with renal disease was not obviously different from normal, although quantification in these patients was difficult because of the relatively small number of glomeruli. PPCs were detected in cases of membranous glomerulonephritis (Fig 23), Bartter's syndrome, Conn's syndrome, mesangial proliferative glomerulonephritis, and renal artery stenosis.

In human kidney, PPCs stained positively for kallikrein. In some cells there was a dense granular staining throughout the cell cytoplasm (Fig 24); in others, individual granules were visible in the cell (Fig 25). In keeping with previous studies, strong immunoreactivity was also seen in segments of the distal tubule (Fig 25). There was weak staining in all proximal tubular cells. No staining was seen in the juxtaglomerular apparatus, including the macula densa, any other glomerular cells, arterioles or interlobular arteries. Preabsorption of the antibody with purified urinary

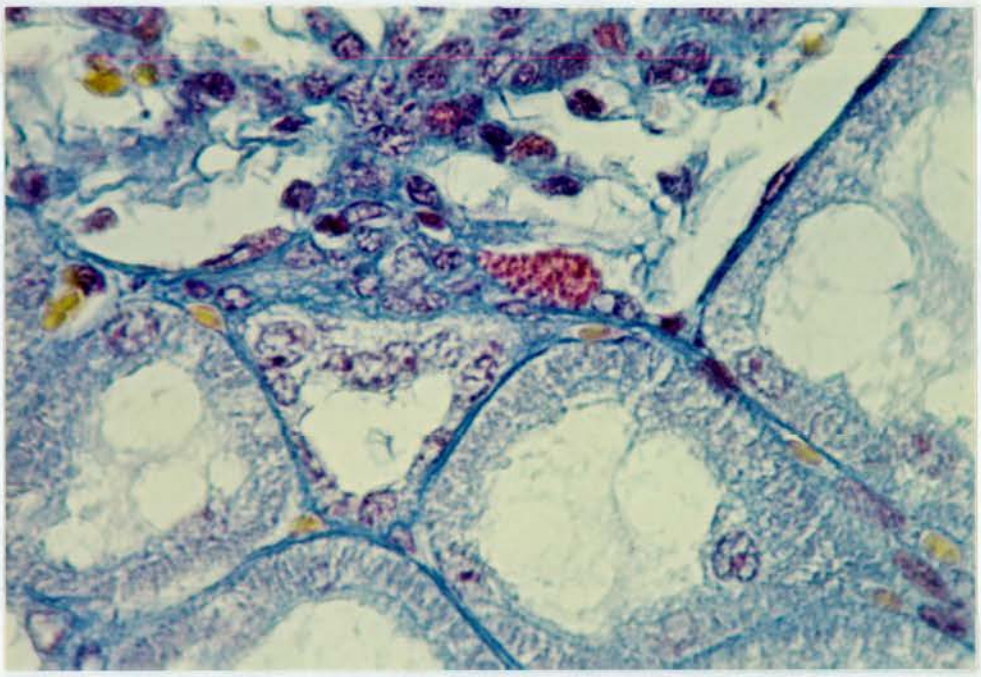


Figure 18. Sheep peripolar cell; Dunn's Crystal Scarlet

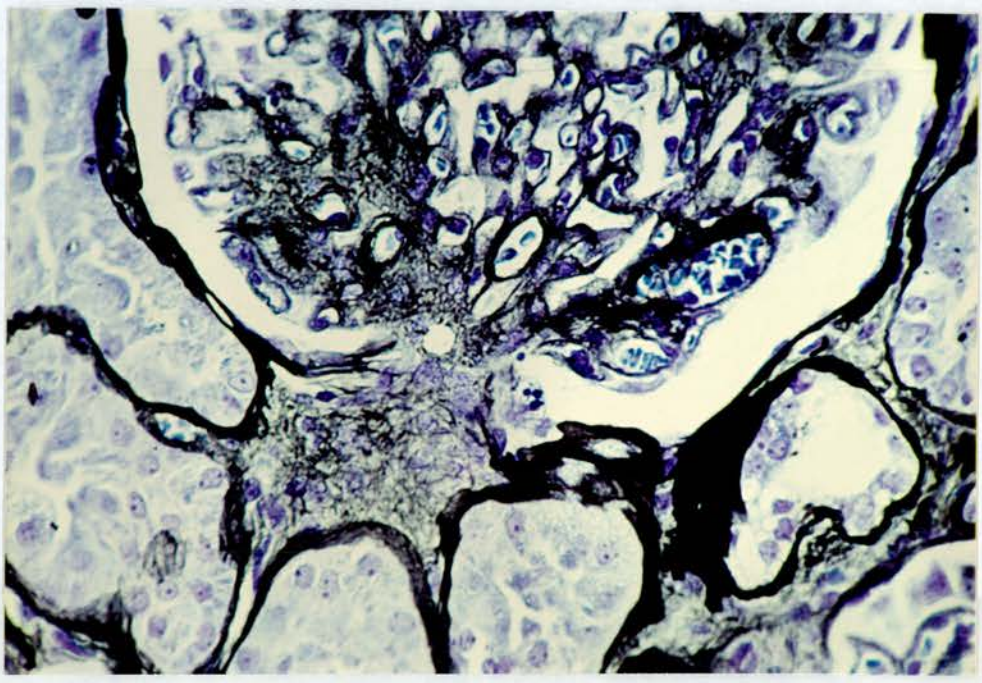


Figure 19. Human peripolar cell; Toluidine Blue

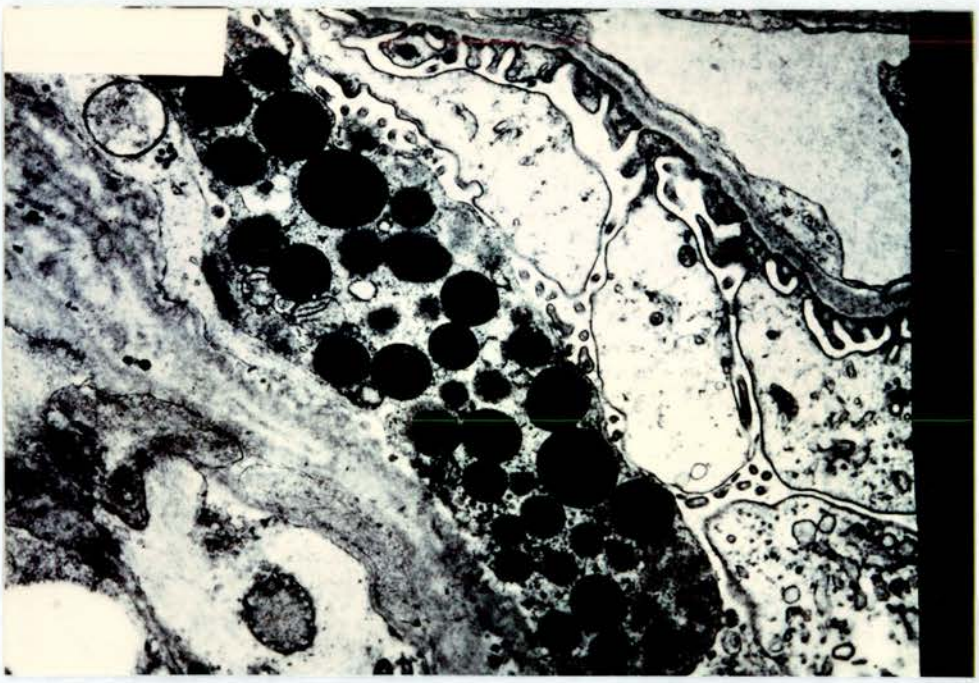


Figure 20. Sheep peripolar cell; electron micrograph

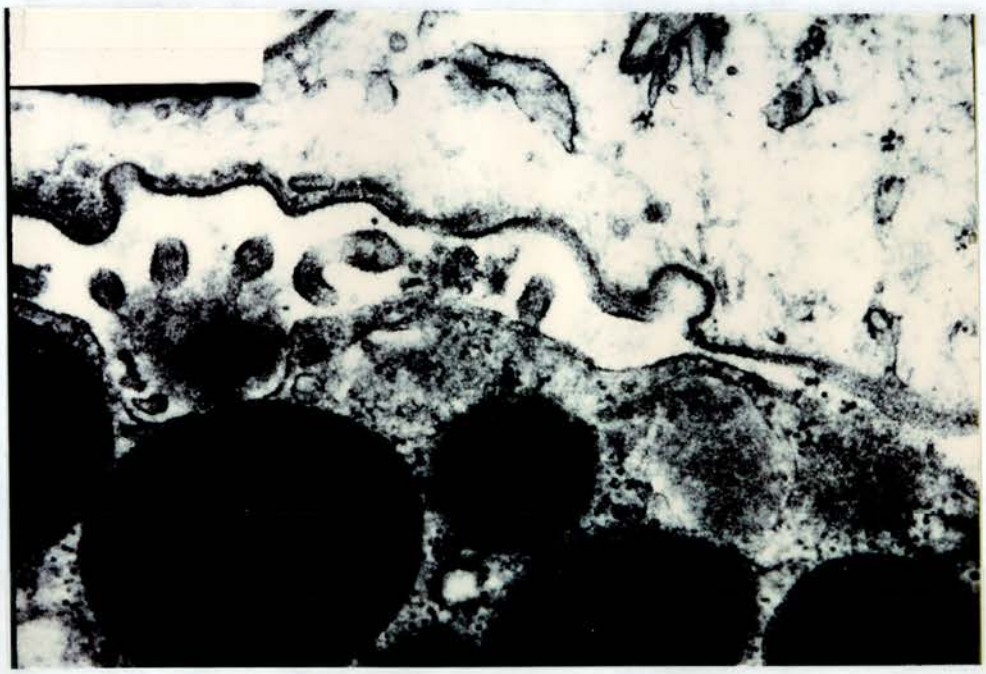


Figure 21. Sheep peripolar cell; exocytosis

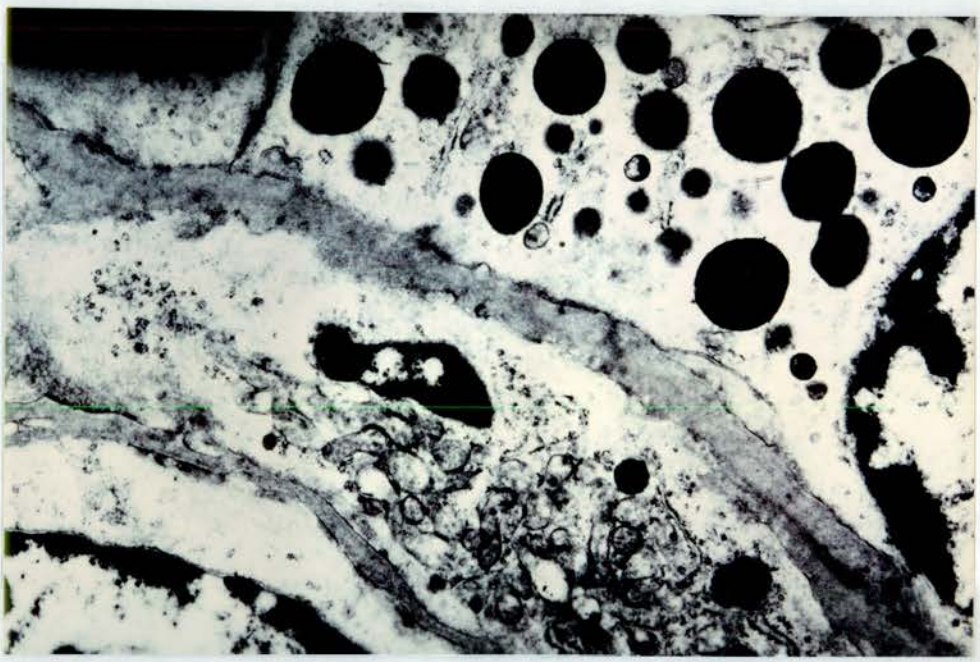


Figure 22. Sheep kidney; peripolar cell and myoepithelial cell separated only by basement membrane

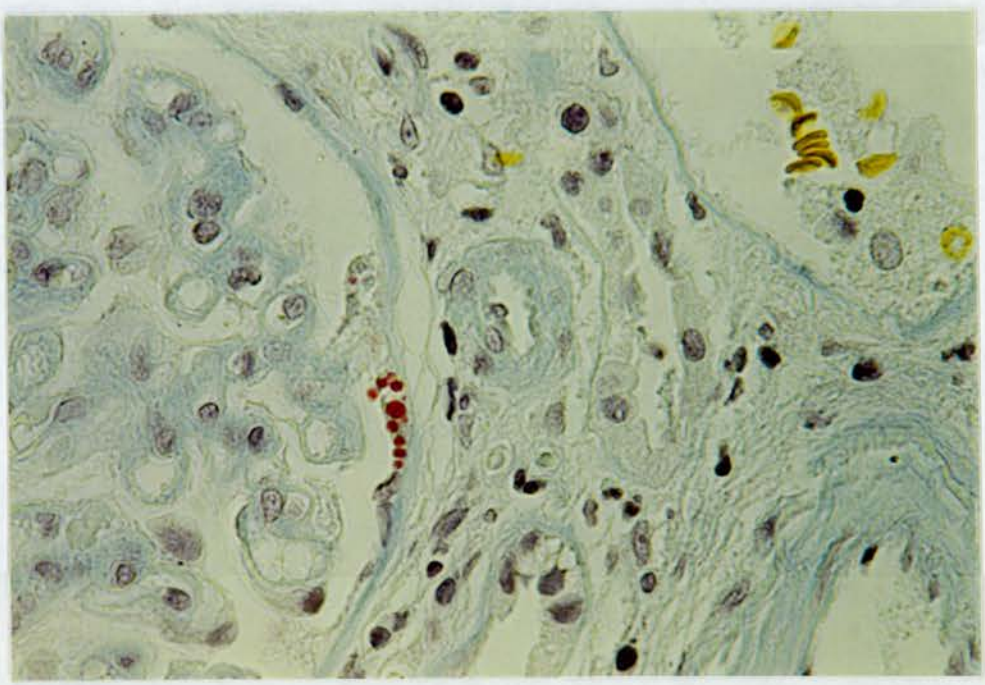


Figure 23. Human kidney, MSB stain. Peripolar cell in a case of membranous glomerulonephritis

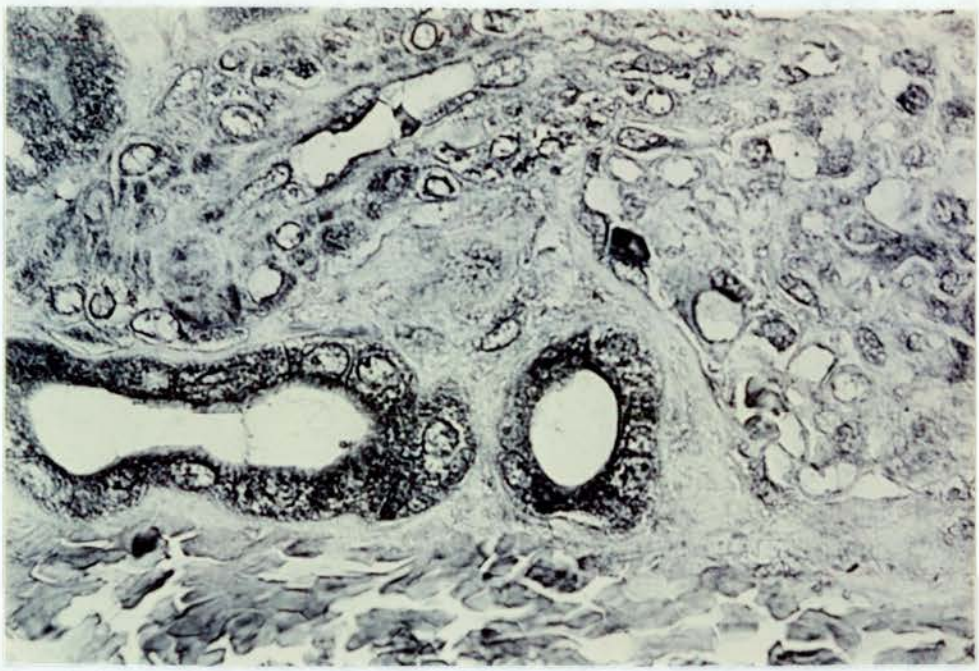


Figure 24. Human kidney, stained with anti-kallikrein antibody (immunoperoxidase technique)

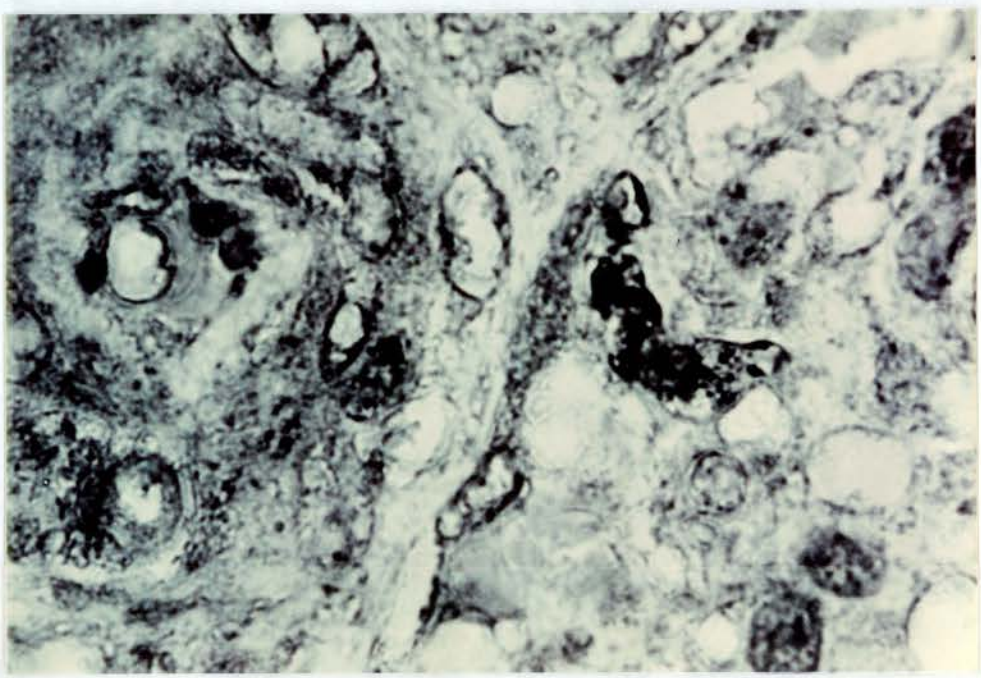


Figure 25. As Fig. 24

kallikrein blocked the staining in PPCs, distal tubular cells and proximal tubules.

5.4. Discussion

Use of special stains, and careful inspection of multiple sections through the vascular pole of the glomerulus, permits recognition of PPCs in glomeruli from both normal and diseased human kidneys. The location of these cells at the vascular pole of the glomerulus affords the potential for influencing glomerular haemodynamics, permeability, and possibly proximal tubular function, and for interacting with other local neuro-endocrine mechanisms. The paucity of organelles for protein synthesis seen in PPCs does not preclude them from being active secretory cells; studies of the kallikrein-secreting granular tubular cell in the rat submandibular gland have shown similar findings under basal conditions, but in response to specific stimuli they generate large amounts of endoplasmic reticulum, which disappears when secretory granules have been restored (Cutter and Chaudry, 1973).

The immunocytochemical studies clearly demonstrate that PPC granules contain kallikrein. This important observation has several implications for the functional role of kallikrein in the kidney. Locally released kallikrein could generate vasodilator kinins which could influence afferent arteriolar tone (Barraclough and Mills, 1965). Kallikrein released into the urinary space could generate kinins which could influence proximal tubular function, both directly and via

generated prostaglandins (Kauker, 1980). In disease states, kinins could increase glomerular permeability, causing proteinuria and changes in glomerular filtration (McFarlane, Mills and Wraight, 1973). Perhaps the most interesting aspect, however, is the potential for interaction with the renin-angiotensin system. It is clear from our results that PPCs and renin-containing cells are frequently in direct apposition. When this is considered in the light of the known biochemical and physiological links between the renal kallikrein-kinin system and the renin-angiotensin system (Pisano, 1975; Sealey, Atlas and Laragh, 1978; Mills, 1972; Proud et al, 1984; Beierwaltes, Prada and Carretero, 1985), and the findings described previously of an apparent influence of the renin/kallikrein ratio on sodium excretion, these findings provide a previously lacking anatomical basis for a close functional association between these two opposing intra-renal mechanisms. Further studies, perhaps involving micropuncture and/or hybridisation histochemistry to assess mRNA synthesis (Coghlan et al, 1985), are required to clarify the role of PPC kallikrein synthesis, and particularly its interactions with renin and Angiotensin II.

CHAPTER 6 URINARY KALLIKREIN EXCRETION IN CHRONIC RENAL FAILURE

6.1. Introduction

In chronic renal failure, renal function is characterised by a high single nephron GFR, elevated glomerular capillary pressure, and high fractional excretion of sodium (Hostetter et al, 1981). The factors mediating these changes are unclear, but increased activity of an intra-renal vasodilator, natriuretic system such as the kallikrein-kinin system would provide a possible explanation. Adetuyibi and Mills found that 24 hour urinary kallikrein excretion was reduced in CRF, but when adjusted for GFR, was in fact increased compared with controls. They suggested that increased kallikrein release by remaining nephrons might increase fractional sodium excretion in CRF, enabling patients to remain in sodium balance (Adetuyibi & Mills, 1972). In contrast, Mitas et al found reduced UKallV in CRF even when adjusted for GFR, and found a negative correlation between UKallV/ml GFR and blood pressure. They suggested that a defect in kallikrein production contributes to hypertension in CRF (Mitas et al, 1978). It was therefore of interest to study the relationship between UKallV, GFR and blood pressure in patients with CRF. The effect of the Angiotensin converting enzyme inhibitor, captopril, was examined in 5 patients with CRF; ACE inhibitors are known to slow the metabolism of kinins, and have been regarded as potentiating the kallikrein-kinin system (Erdos, 1976).

6.2. Patients and methods

22 patients with CRF were studied (creatinine clearance 15 ± 13 ml/min, mean ± SD). 9 were normotensive (mean arterial pressure [MAP] <105 mm Hg) and 13 hypertensive (MAP >105 mm Hg). 11 healthy controls were studied; all were normotensive, on no drug therapy, with normal serum creatinine and creatinine clearance values and negative urinalysis. Details of patients and controls are shown in Table 6. All subjects were on an unrestricted intake of salt and water; no diuretics or anti-hypertensive drugs were given for at least 48 hours before study. Two 24 hour urine collections were taken and the mean value used for analysis. In the captopril component of the study, 5 patients with CRF and hypertension were given captopril (Capoten, Squibb) orally for 5 days, after a 72 hour period off drugs. Initial dose was 12.5 mg daily, increasing as necessary to control blood pressure (maximum dose 75 mg daily). Patient details are in Table 7.

6.3. Results

Compared with controls, UKallV was reduced in both groups of CRF patients (controls 12.4 ± 5.2 nkat/day, normotensive 2.9 ± 1.9 nkat/day, hypertensive 3.7 ± 2.8 nkat/day; $p<0.01$ for both groups versus controls)(Fig 26). There was no difference between normotensive and hypertensive patients, and no significant correlation between UKallV and blood pressure. When the kallikrein excretion rate was divided by the GFR, as measured by endogenous creatinine clearance,

Sex	Age (yrs)	Diag-nosis	Ccreat ml/min	Blood pressure (mm Hg)	U.Vol L/day	U NaV mmol /day	UkallV nkat /day	UkallV /mlCcr
Normotensive								
M	56	PAN	12.9	130/70	1.70	90	4.7	.364
F	56	MM	5.5	150/80	2.76	114	3.4	.586
M	50	CP	4.6	150/80	2.14	103	3.1	.674
F	67	MCGN	26.4	150/80	1.12	97	1.3	.049
M	55	Amyl	29.8	120/70	1.90	95	3.1	.104
F	40	CP	4.4	120/70	1.27	73	0.6	.138
F	70	Memb	53.4	150/80	0.90	72	5.4	.118
M	65	IN	4.3	150/70	1.50	133	4.6	1.07
F	46	CP	0.6	130/80	2.80	122	0.2	.333
Mean								
±SD	56.1		15.8	138±13	1.51	99	2.93	0.38
	±9.9		±17.5*	75±5	±0.73	±21	±1.87*	±0.34*
Hypertensive								
F	69	GS	12.5	160/90	1.27	68	0.6	.077
M	67	GS	15.5	180/110	1.83	109	4.5	.290
M	34	PGN	14.0	180/110	1.78	78	4.4	.314
M	38	MCGN	10.0	180/110	1.68	118	0.7	.074
M	44	HTNS	6.9	160/100	1.38	95	2.8	.406
M	53	HTNS	29.6	200/140	1.40	108	5.	.198
F	51	HTNS	7.9	190/120	1.47	13	6.7	.815
F	45	Amyl	1.4	170/100	0.80	52	0.7	.500
M	60	GS	9.2	160/90	2.56	102	1.8	.192
F	40	PGN	11.0	160/100	2.79	146	4.5	.409
M	33	PGN	27.0	220/130	3.08	129	10.3	.382
F	64	HTNS	21.9	240/120	2.16	143	1.8	.092
F	63	CP	43.6	160/90	1.55	121	3.2	.077
Mean								
±SD	50.9		16.2	182±25*	1.83	98	3.68	0.29
	±12.8		±11.5*	108±16*	±0.65	±38	±2.81*	±0.21*
Controls								
M	36		108	120/80	1.88	157	8.7	.081
F	30		112	130/80	1.49	153	16.0	.143
M	36		95	120/85	1.67	178	6.2	.065
F	67		102	130/80	1.78	138	16.4	.161
F	59		94	110/70	0.94	130	7.2	.077
F	42		114	125/80	1.54	129	10.0	.088
M	38		115	120/85	2.08	159	24.5	.213
M	61		101	130/85	1.73	169	10.0	.099
F	61		109	135/80	0.76	84	14.6	.134
F	39		119	120/80	1.20	150	11.7	.098
M	44		98	125/80	1.10	77	11.0	.112
Mean								
±SD	46.6		106.1	124±7.0	1.47	138	12.4	0.12
	±12.8		±8.6	80±4.2	±0.42	±32	±5.2	±0.04

TABLE 6 Details of 22 patients with chronic renal failure and 11 controls.
*different from controls ($p < 0.05$)

Table 6; abbreviations

U.Volume - urine volume
UKallV - 24 hour urinary kallikrein excretion
CCr - creatinine clearance
PAN - polyarteritis nodosa
MM - multiple myeloma
Amyl - amyloid
CP - chronic pyelonephritis
MCGN - mesangiocapillary glomerulonephritis
Memb - membranous glomerulonephritis
GS - glomerulosclerosis ? cause;
PGN - proliferative glomerulonephritis
HTNS - hypertensive nephrosclerosis
IN - chronic interstitial nephritis

Name	Sex	Age (years)	Diagnosis	Ccreat (ml/min)	Pcreat(umol/l) (pre-C)	(on C)
AP	M	34	PGN	14	826	870
WM	M	33	PGN	27	316	414
MJ	F	51	HTNS	20	611	628
AM	M	53	HTNS	20	235	286
MR	M	58	HTNS	18	412	514
Mean±SD		46±11.5		19.8±4.7	480±239	542±222*

Name	BP (mmHg) (pre-C)	(on C)	PRA (ng/ml/hr) (pre-C)	(on C)	UKallV (nkat/24h) (pre-C)	(on C)
AP	180/110	150/100	6.1	24.1	4.68	2.45
WM	220/130	150/100	26.8	42.7	10.35	3.40
MJ	190/120	160/80	15.1	30.8	6.25	0.80
AM	200/140	140/90	2.3	59.2	5.92	1.15
MR	220/130	140/90	36.0	67.6	6.24	1.35
Mean	202+18	148+8*	17.3	44.9	6.69	1.83
±SD	126±11	92±8*	±14.1	±18.4*	±2.15	±1.07*

Table 7 Details of 5 hypertensive patients with chronic renal failure given captopril.

Abbreviations:
Ccreat - creatinine clearance
Pcreat - plasma creatinine
PRA - plasma renin activity
PGN - proliferative glomerulonephritis
HTNS - hypertensive nephrosclerosis
BP - blood pressure
C - captopril

* $p < 0.05$ compared with pre-captopril

values were increased in CRF compared with controls (controls 0.12 ± .04, normotensive 0.38 ± 0.34, hypertensive 0.29 ±0.21; $p<0.05$ for both groups versus controls)(Fig 27). Results were similar in normotensive and hypertensive patients, and there was no correlation with blood pressure. Neither UkallV nor UkallV/ml GFR correlated with age, creatinine clearance, urine volume, or urinary sodium excretion ($p>0.05$). In the captopril study, all patients showed a fall in blood pressure and a rise in PRA after treatment (Table 7). There were no consistent or significant changes in urine volume or sodium excretion on captopril, but all subjects showed a small increase in plasma creatinine after 5 days (Table 7). On captopril, UKallV fell in all patients, and was significantly reduced ($p<0.05$) on Day 4 and Day 5 of therapy (Fig 27a).The fall in urinary kallikrein excretion tended to coincide with effective lowering of blood pressure, and there was a positive correlation between MAP and UKallV on captopril (Fig 28).

6.4. Discussion

The function of the remaining nephrons in CRF is the subject of intense investigation at present. It has been suggested that the hyperfiltration and increased glomerular capillary pressure may contribute to the progression of CRF (Hostetter & Brenner, 1981); prostaglandins and thromboxanes have been implicated as chemical mediators of this effect (Bergman et al, 1986). Since kinins are potent vasodilator substances and stimulate arachnidonic acid metabolism, increased

Fig.26 24 hour urine kallikrein excretion in 11 controls and 23 patients with chronic renal failure (CRF)

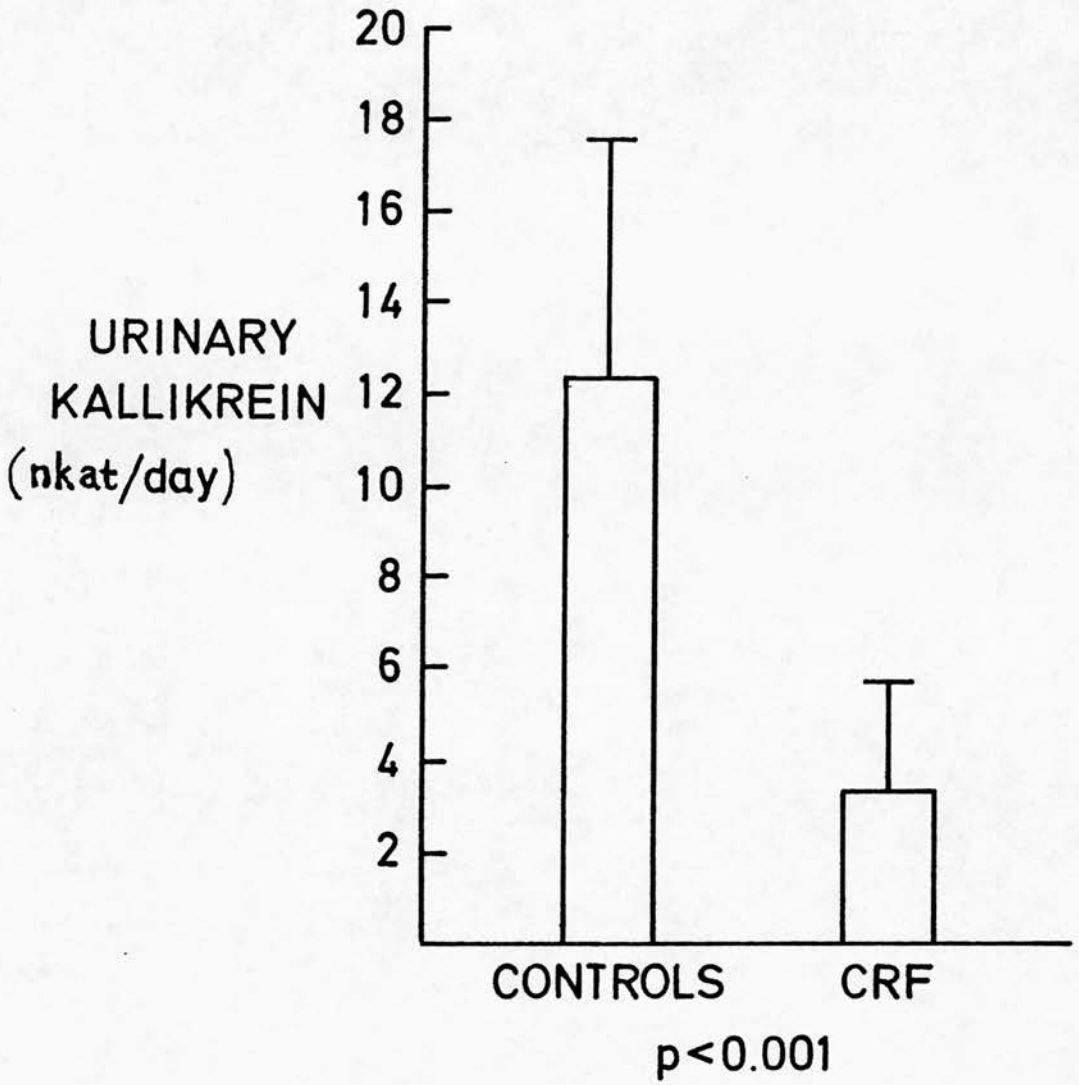


Fig. 27 U_{KallV} corrected for creatinine clearance in 11 controls and 23 patients with CRF

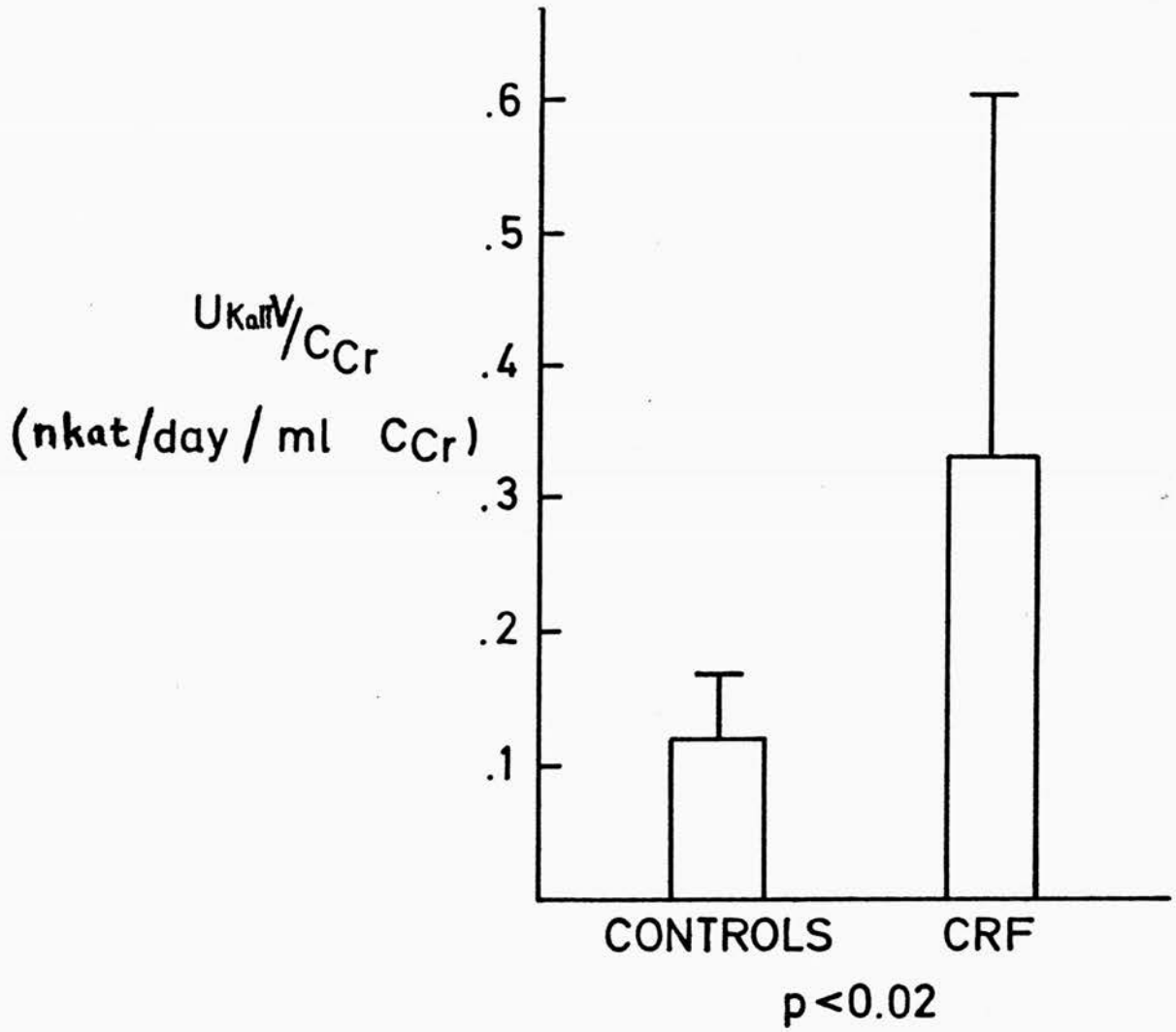


Fig.27a Mean urine kallikrein excretion in 5 patients with CRF and hypertension, in relation to Captopril therapy

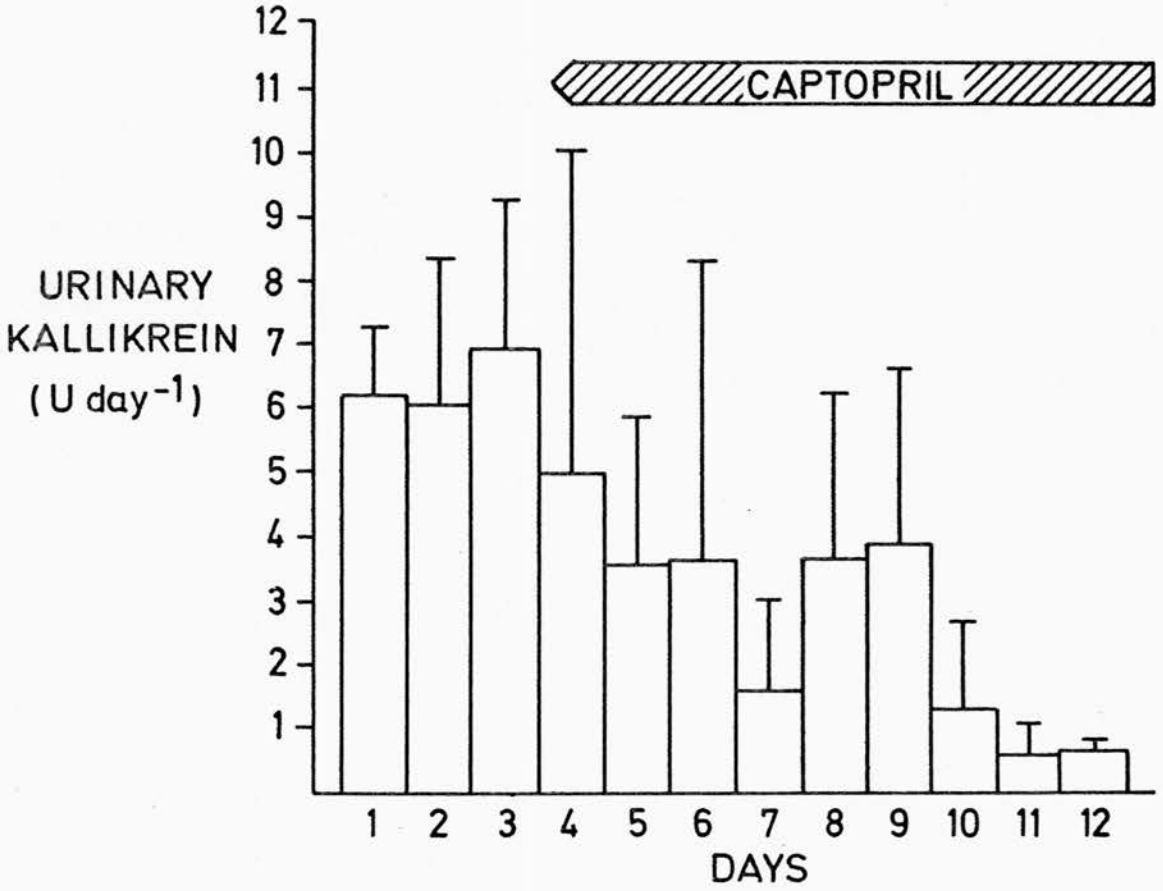
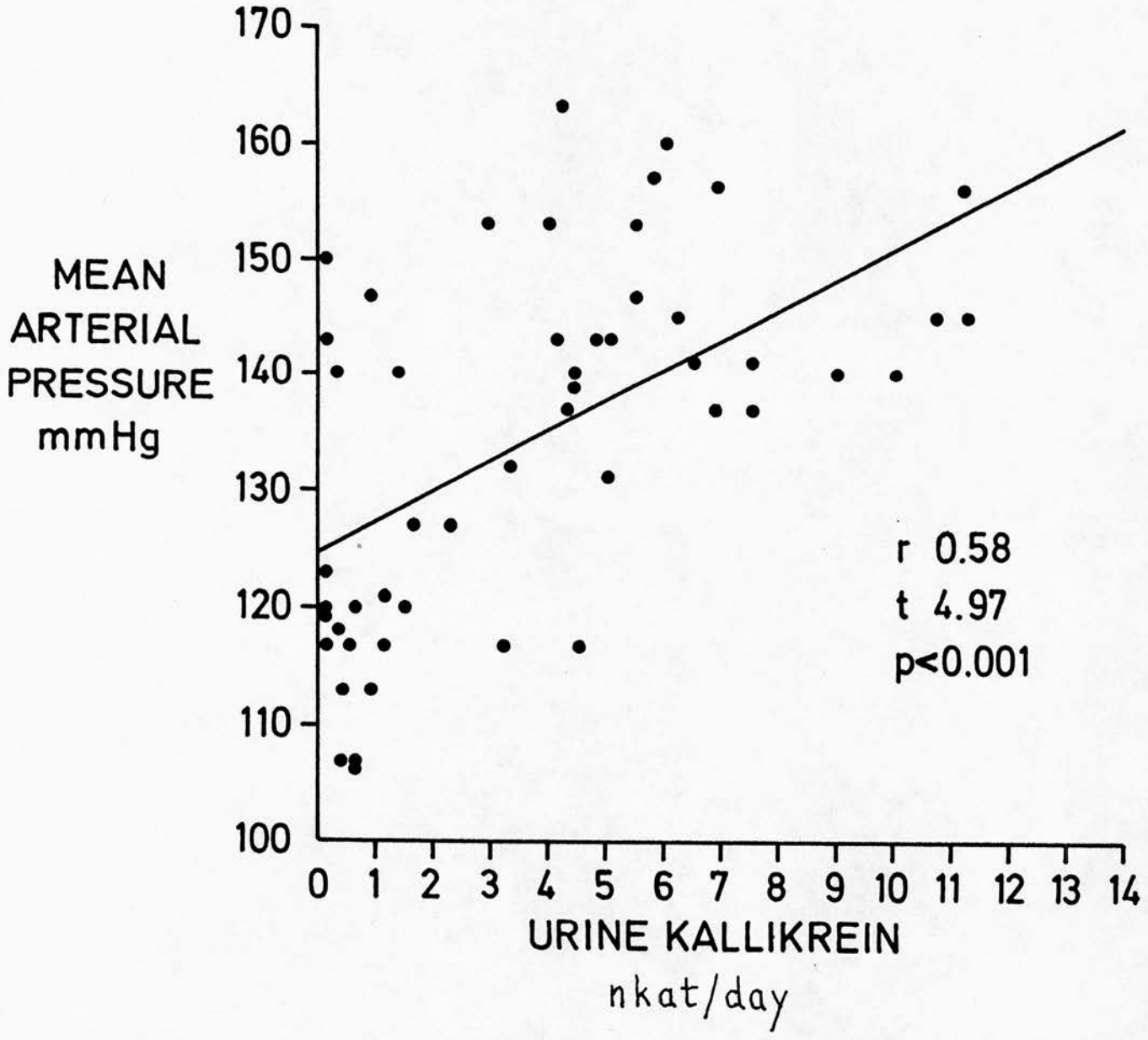


Fig.28 Correlation between daily mean arterial pressure and urine kallikrein excretion in 5 patients given Captopril



activity of the kallikrein-kinin system in remaining nephrons could contribute to this mechanism of progression also. In order to preserve sodium balance in face of a low whole-kidney GFR, tubular reabsorption of sodium must fall, leading to an increased FENa. A natriuretic effect of kinins at a tubular level could be implicated in this change also (Kauker et al, 1980). While absolute excretion of kallikrein is decreased in CRF, the reduction in UKallV is proportionally less than the fall in GFR, as measured by creatinine clearance. When adjusted for GFR, UKallV is increased 2-3 fold in CRF. Since kallikrein in urine is derived from cells of functioning nephrons (Orstavvik et al, 1976), one would predict that in the absence of altered stimuli to kallikrein release, kallikrein in the urine of patients with CRF would be reduced either in parallel, or in excess of, the decline in GFR, depending on the degree of hyperfiltration by remaining nephrons. The above observations therefore suggest that kallikrein release by remaining nephrons is increased in CRF. The location of kallikrein in glomerular peripolar cells, described above, increases the possibility of kallikrein-kinins influencing glomerular and proximal tubular function in this context. No experimental study has yet examined the effect of kallikrein or kinin inhibition in CRF, either in an animal model or in a clinical setting. Such studies could shed important light on the pathogenesis of altered glomerular and tubular function in CRF.

The pathogenesis of hypertension in CRF is undoubtedly

complex and multifactorial. Unlike other studies, no significant link was observed between blood pressure and kallikrein excretion in CRF (Adetuyibi & Mills, 1972, Mitas et al, 1978, Abe et al, 1981). It remains possible that the overall balance between pressor and depressor influences is disturbed in CRF, contributing to hypertension in some cases, while others may be more affected by disturbed volume homeostasis. More detailed analysis of the correlation between the renal kallikrein-kinin system and other vasoactive hormone systems may prove informative, as has been found in essential hypertension. (Abe et al, 1981, Pierucci et al, 1985, Levy et al, 1977).

The mechanism whereby captopril lowers kallikrein excretion in CRF is unknown (Marin-Grez, Bonner & Gross, 1980). Angiotensin converting enzyme in fact has a higher affinity for bradykinin than for angiotensin I (Erdos, 1975). If kallikrein release from submandibular glands is stimulated in animals given captopril, profound hypotension ensues (Scicli et al, 1983). It therefore seems likely that given a constant degree of kallikrein release, ACE inhibition potentiates the kallikrein-kinin system. This effect could, however, be negated or reversed by a concomitant reduction in kallikrein release, as suggested by the results of this study. A negative feedback system, whereby increased circulating kinins supress renal kallikrein release, has been proposed as an explanation of the effect of captopril on UKallV (Cunningham & Brouhard, 1980, Karlberg et al

1980); this seems unlikely, since infusion of bradykinin into the renal artery increases kallikrein excretion (Mills, Newport & Obika, 1979). It has been suggested that aldosterone is an important modulator of kallikrein release, and plasma aldosterone falls during captopril therapy (Geller et al, 1972). In the isolated perfused kidney there is a direct correlation between renal perfusion pressure and UkallV (Bevan, MacFarlane and Mills, 1974); the reduced systemic blood pressure on captopril could therefore have contributed to the reduction in UKallV. This is supported by the observed correlation between kallikrein excretion and MAP while on captopril, as shown in Figure 29.

It is of interest that ACE inhibitors have recently been shown to slow the progression of chronic renal failure, even at an advanced stage (Heeg, De Jong & De Zeeuw, 1989); the possibility that this is in part due to decreased renal kallikrein-kinin activity merits investigation. Urinary kallikrein excretion is inappropriately reduced in some types of acute renal failure (Funaki et al, 1982), and captopril is known to cause acute renal failure, particularly in states of reduced renal perfusion (Hricik et al, 1983). All the patients in this study showed some increase in plasma creatinine in the short term. This acute response could also relate to decreased renal kallikrein-kinin activity. Overall it seems likely that the renal kallikrein-kinin system is involved in the renal respone to ACE inhibition, and that the conventional concept of a stimulatory effect may be erroneous.

CHAPTER 7. THE RENAL KALLIKREIN-KININ SYSTEM IN THE NEPHROTIC SYNDROME

7.1. The nephrotic syndrome (NS) can occur in various forms of renal disease; it is characterised by proteinuria, usually >4 g/day, sufficient to cause hypoalbuminaemia, and, in the untreated state, by the presence of peripheral oedema. Two aspects of this disease state remain problematical. Firstly, the pathophysiology of the profound glomerular protein leak is often unclear (Brenner, Hostetter and Humes, 1978). In many cases, there is obvious disruption of the glomerular basement membrane by deposits of immune material, eg in membranous glomerulonephritis. In other cases, however, structural damage to renal tissue may be slight or absent, eg in minimal change nephropathy. Subtle changes in the surface charge of the glomerular basement membrane have been blamed for the increase in capillary permeability (Olson, Rennke and Venkatacham, 1981), but other authors have postulated that a local or systemically-derived permeability factor contributes to the protein leak. Permeability factors derived from lymphocytes and from mast cells have been described (Lagrue et al, 1975; Strauss, Freundlich and Zillevelo, 1984). The possibility that renal kallikreins and kinins might play a role in the pathogenesis of NS was first suggested in a paper by Murakami in Nature in 1968. He showed that infusion of glandular kallikrein into the renal artery of dogs caused heavy proteinuria. This could be prevented by the concomitant administration of

aprotonin and an anti-histamine (mepyramine), which was of interest because of the known synergy between the effects of histamine and kinins on capillary permeability (Murakami, Hori and Masamura, 1968). In 1979, Brouhard described increased urinary kallikrein excretion in children with minimal-change nephropathy, which returned to normal when remission was induced with steroids (Brouhard et al, 1979). In subsequent correspondence, however, this phenomenon was ascribed to the association between high plasma aldosterone concentrations and increased urinary kallikrein excretion which had been observed in the physiological studies of Margolius and others (Geller at al, 1972; Margolius, 1980). It was assumed that all NS patients were likely to have secondary aldosteronism, as a result of hypovolaemia, and that the increased UKall was merely a marker of this. However, no measurements were made to confirm this assumption, and no further studies of the significance of the observation were made.

Without diuretic treatment, most patients with NS retain sodium, although the degree of sodium retention varies considerably between cases (Strauss, Freundlich and Zillervelo, 1984). Traditionally this was ascribed to a reduction in circulating plasma volume, consequent to the reduced plasma oncotic pressure (Dourhout-Mees et al, 1979). Hypovolaemia was thought to stimulate the renin-angiotensin-aldosterone axis, causing an increase in tubular sodium reabsorption. It seems clear that such a mechanism does operate in some cases of NS (Meltzer et al, 1979). Equally,

it is now clear that in others, the overall expansion of extracellular fluid volume is such that plasma volume is normal, or even increased, and that plasma renin activity is normal or suppressed (Brown et al, 1982). In this "hypervolaemic" type of NS, the reason for continued renal retention of sodium is unclear; evidence supports an as yet unidentified intra-renal defect in sodium excretion (Firth, Raine and Ledingham, 1989).

It was therefore of interest to investigate the activity of the renal kallikrein-kinin system in NS, both as a possible mediator of increased capillary permeability, and as a factor in the intra-renal regulation of sodium excretion.

7.2. PATIENTS AND METHODS

7.2.1. Part 1

To clarify the relationship between NS and kallikrein excretion, the first part of the study examined urinary kallikrein excretion in 44 subjects. 32 were patients with histologically proven glomerulonephritis (GN), 16 of whom had NS (proteinuria >4 g/day, hypoalbuminaemia, and oedema). 12 healthy volunteers were controls (8m, 4f, mean age 41 $\pm$ 13 years). Age and sex were similar in each group. Details of patients are shown in Table 8. Patients were not receiving diuretics or drugs known to influence kallikrein excretion. Two 24-hour urine collections were obtained from each subject, while taking an 100-200 mmol sodium diet and unrestricted oral fluids. Patients were studied in hospital on a supervised diet; controls were not hospitalised but

	Sex	Age	Histology	Ccreat ml/min	Proteinuria g/24h	Plasma albumin g/l	Urinary kallikrein nkat/24h
Glomerulonephritis, not nephrotic							
	M	55	PGN	13	2.5	32	11
	F	68	PGN	10	2.5	36	0.6
	M	66	PGN	5	4.6	30	4.5
	M	33	PGN	14	2.0	42	4.4
	M	53	PGN	100	3.7	39	7.9
	M	37	MCGN	10	15	32	0.7
	F	55	PGN	5	5	31	3.4
	F	49	PGN	4	0.5	32	3.5
	M	43	PGN	10	2.0	43	2.8
	M	27	PGN	20	<0.3	40	5.9
	F	50	PGN	20	2.0	40	6.2
	F	66	MCGN	40	1.0	33	1.3
	F	54	PGN	30	1.1	32	3.0
	M	16	PGN	140	<0.3	40	6.8
	F	55	PGN	15	3.0	36	4.5
Mean ± SD		49±14		29±38	2.9±3.6	36.3±4.6	4.6±2.8
Glomerulonephritis, nephrotic syndrome							
	M	75	memb	10	25	20	35
	M	47	MCGN	10	14	24	33
	F	15	PGN	30	10	30	12
	F	69	PGN	7	10	23	17
	M	16	ML	100	11	23	31
	M	51	memb	100	30	21	35
	F	20	memb	120	5	28	9.5
	M	19	MCGN	30	30	23	19
	M	78	memb	50	10	24	20
	M	74	MCGN	45	10	29	32
	M	42	PGN	60	35	20	28
	F	21	memb	80	17	22	41
	M	26	ML	120	8	20	27
	F	25	MCGN	15	8	28	43
	M	27	PGN	30	12	24	21
	M	65	MCGN	40	11	16	21
Mean ± SD		42±24		53±39	15.4±9	23.4±3.7	26.7±9.9

Table 8 Details of 32 patients with glomerulonephritis

PGN = proliferative glomerulonephritis
MCGN = mesangiocapillary glomerulonephritis
ML = minimal lesion glomerulonephritis
memb = membranous glomerulonephritis
Ccreat = creatinine clearance

were instructed to take 100-200 mmol sodium daily; urine sodium excretion was measured in all subjects.

Since active kallikrein might become bound to protein in urine, affecting the results in heavy proteinuria, a solution of human albumin, 43 g/l, was added to four control urine samples, to a protein concentration equivalent to nephrotic urine (10 g/l). Kallikrein activity was compared with and without protein in each sample.

To investigate the possibility of leakage of kallikrein from plasma, citrated plasmas from 5 nephrotic patients were incubated with the chromogenic substrate, as in the urine assay, and activity compared with urine from the same patient.

PRA and plasma aldosterone concentration were measured by established radioimmunoassay methods in 5 patients with NS (Roulston et al, 1983; Al-Dujaili and Edwards, 1978).

7.2.2. Part 2

In this part of the study, examining further the relationship between PRA and UKallV in NS, we studied 16 patients with NS and 22 healthy controls. Details of subjects are shown in Table 9; all had greater than 4G proteinuria per day and plasma albumin below 30 G/L. The mean age and male/female ratio were similar in each group, except for the fact that patients in the normal renin nephrotic group were significantly older than those in the high-renin group, but not significantly older than the controls (Table 9). Patients and controls were studied

Sex	Age yrs	Histology	C Cr ml/min	Uprot g/24h	Palb g/l	UNaV mmol /24h	UKV mmol /24h	PRA ng/ml /hr	U KallV nkat /24h
High renin									
M	16	ML	97	11	17	47	49	11.6	31
M	51	memb	95	20	24	75	50	3.0	35
F	46	MP	78	15.2	22	2	38	35.6	29.9
M	40	ML	84	17	18	2	56	12.8	66.5
F	21	memb	80	17	23	75	84	10.8	41.5
F	51	memb	78	4.7	23	28	52	3.6	20.7
M	16	ML	120	12	17	21	65	14.5	48.6
M	30	MCG	20	25	22	97	60	4.6	49.3
Mean	33.8		81.5~	15.2~~	20.8~~	43.4~~	56.8	12.1~~	40.4~~
SEM	5.3		10.1	2.2	1.0	12.7	4.8	3.7	5.1
Normal renin									
F	73	MP	59	4.9	25	40	27	1.8	29.0
M	65	MCGN	46	11	17	52	48	.64	23.9
M	74	MCGN	16	16.2	27	59	73	.50	35.1
M	35	MP	123	7.9	20	77	54	.59	22.0
F	37	ML	95	6.8	20	64	74	.92	34.4
F	73	MCGN	20	5.1	27	45	22	.10	17.1
F	69	memb	19	5.6	18	33	36	2.7	18.9
M	41	MP	63	16	23	40	30	2.3	25.0
Mean	58.4*		55.1~	9.2~~	22.1~~	51.3~~	45.5	1.2**	25.7~*
SEM	6.2		13.7	9.2	1.4	5.2	7.2	.30	2.4
Normals (n=22)									
Mean	44.2	m13	110	<0.3	40.2	121	60.5	1.45	12.0
SEM	7.2	f9	3.6		1.46	3.2	8.9	.22	1.1

~ p<.05 different from normals
~~ p<.01 " " "
* p<.05 different from high renin group
** p<.01 " " " " "

Table 9
Urinary kallikrein-PRA study. Details of subjects with high-renin nephrotic syndrome (n=8), normal-renin nephrotic syndrome (n=8) and healthy controls (n=22).

memb - membranous; ML - minimal lesion; MP - mesangial proliferative; MCGN - mesangiocapillary; C Cr - creatinine clearance; Uprot - urinary protein excretion; Palb - plasma albumin; UNaV - urinary sodium excretion; UKV - urinary potassium excretion.

under similar conditions to Part 1 of the study. Mean daily urinary sodium excretion in the control subjects was 121 ± 3.2 mmol/day (Table 9). UKallV was measured in two consecutive 24-hour urine collections and a mean of the two values calculated. Blood for PRA estimation was taken at 8 am after the subject had been supine for one hour. Plasma volume was measured in 8 of the patients, by estimating the volume of distribution of I125-labelled radio-albumin over one hour. Normal values for this assay have been established previously in 20 healthy subjects (International Commitee for Standardisation in Haematology, 1973). In the NS subjects, the volume/weight adjustment was made on the basis of a "dry" weight, established after subsequent abolition of oedema by diuretic therapy. On average, 1.8% of the total administered radioactivity appeared in urine during the 1 hour study.

In order to assess the possibility of glandular kallikrein reaching the systemic circulation in NS, we compared the activity of plasma against glandular kallikrein substrate in 10 patients with NS, 17 patients with glomerulonephritis who were not nephrotic, and 10 normal subjects. Samples were taken at 8 am after overnight fast. Details of these subjects are shown in Table 10. Age and sex ratio were similar in the three groups; creatinine clearance was significantly lower and urinary protein excretion higher than the controls in the NS and glomerulonephritis groups. Urinary protein excretion was significantly greater and

Sex	Age yrs	Histology	C Cr ml/min	UProt g/24h	P Alb g/l	P Gl Kall absx10-3
Nephrotic syndrome						
M	40	ML	84	17	18	10
M	16	ML	120	12	17	0
F	69	memb	19	5.6	18	5
M	30	MCGN	20	25	22	12
F	15	ML	67	4.1	19	8
F	73	MP	59	4.9	25	11
M	65	MCGN	46	11	17	23
M	35	MP	123	7.9	20	0
F	61	MP	99	4.1	29	8
M	45	MCGN	60	8.2	21	6
Mean	44.9		69.7~	10~~**	20.6~~**	8.3~~*
SEM	6.8		11.7	2.1	1.2	2.09
Glomerulonephritis, no NS						
M	25	FNP	38	2.3	32	0
M	31	MCGN	110	.5	38	0
M	42	MP	90	.9	46	1
M	49	memb	15	2.6	41	2
F	55	MP	64	<.3	41	2
F	69	MCGN	19	2.5	35	0
F	50	MP	25	1.5	45	0
M	24	MP	110	.6	48	4
M	59	MP	62	1.4	39	0
M	50	FNP	21	2.2	39	3
F	20	MP	102	.8	38	3
F	22	MP	103	.5	40	0
F	16	MP	110	.8	44	2
M	36	MP	122	1.2	40	4
F	32	MP	69	1.3	46	3
M	27	memb	120	3.5	32	0
M	16	MP	87	.6	40	1
Mean	36.7		74.5~	1.4~~	40.2	1.47
SEM	3.9		9.3	.2	1.1	.36
Normals (n=10)						
Mean	40.4	6m 4f	108.3	<.3	41.5	1.7
SEM	6.1		6.4		.65	.45

~ p<.05 different from normal
~~ p<.01 " " "
* p<.05 different from GN
** p<.01 " " "

FNP = Focal necrotising proliferative; P Gl Kall - plasma glandular kallikrein activity

Table 10
Plasma glandular kallikrein study; details of patients with nephrotic syndrome (n=10), glomerulonephritis without nephrotic syndrome (n=17) and healthy controls (n=10)

plasma albumin lower in the nephrotic group than the glomerulonephritis group.

In this part of the study , activity of plasma against S2266 was measured using the method of Gallimore et al for estimation of "kallikrein-like" activity in plasma (Gallimore and Friberger, 1982; Chapter 2, Section 2.3.2). The method was as previously described, except for the replacement of S2302, a specific substrate of plasma kallikrein, by S2266, the glandular and renal kallikrein substrate. 50 µl of plasma was diluted with 3000 ul buffer, then incubated for 2 minutes at 37°c with a further 200 µl buffer. 200 µl of S2266 was then added. After 2 minutes incubation at 37°c, 200 µl of 50% acetic acid was added. The absorbance was measured at 405 nm; from this was subtracted the absorbance of a blank prepared by adding the same reagents in reverse order.

7.3. RESULTS

7.3.1. Part 1

As shown in Fig 29, UKallV was reduced in GN compared with controls, but was increased in NS. Only two NS patients had a value within 1 SD of the mean in controls. Urine volume was similar in each group (controls 1.47 ± 0.44 l/day, GN 1.69 ± 0.53 l/day, NS 1.28 ± 0.54 l/day). Urinary sodium excretion was lower than the controls in GN and NS (controls 150 ± 47 mmol/day, GN 109 ± 57 mmol/day, NS 85 ± 42 mmol/day), but only the control/NS difference was significant ($p<0.001$). Creatinine clearance was

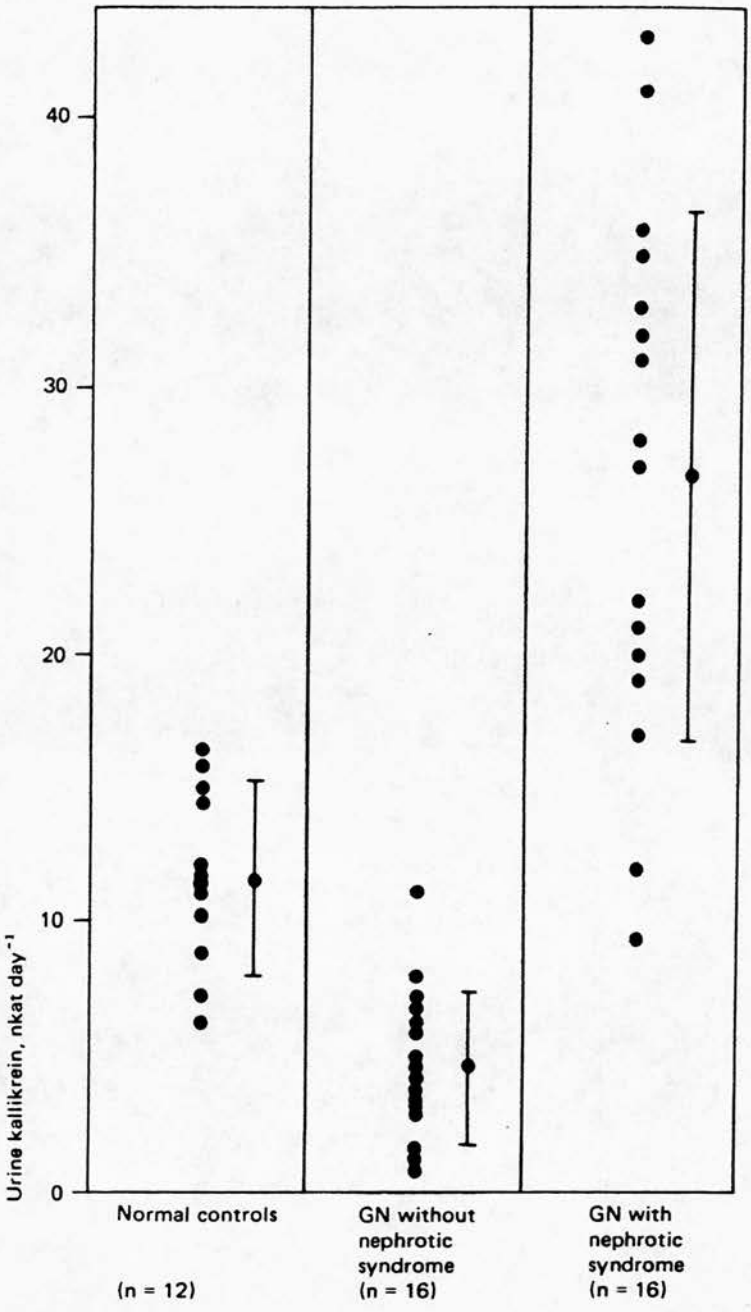


Fig. 29. Daily excretion of urinary kallikrein in normal controls, patients with GN and patients with both GN and nephrotic syndrome.

significantly lower than controls in GN and NS (Table 8). UKallV did not correlate with urine volume, urinary sodium, or creatinine clearance. In the 32 patients with renal disease, UKallV correlated positively with urinary protein excretion (r 0.65, $p<0.001$) and negatively with plasma albumin (r-0.73, $p<0.001$).

When corrected for dilution, urinary kallikrein concentration in control urines was not affected by addition of albumin (control 6.20 $\pm$ 0.52 U/l, urine + albumin 6.81 $\pm$ 0.42 U/l, $p>0.05$).

Incubation of NS patients plasma with the chromogenic substrate showed activity averaging 17.5 $\pm$ 9.3% of the corresponding urine value (Table 11).

In the 5 NS patients in whom PRA and aldosterone were measured, PRA was normal in 4 and slightly elevated in 1, compared with the laboratory reference range (mean 1.92 $\pm$ 1.15 ng/ml/hr, normal range 0.2 - 2.7 ng/ml/hr). Plasma aldosterone was normal or low in all 5 (mean 37 $\pm$ 17 pg/ml, normal range 50 - 140 pg/ml). Kallikrein excretion was markedly elevated in all 5 patients (mean UKallV 27 $\pm$ 8.9 nkat/day).

7.3.2. Part 2

Of the 16 patients with NS in this part of the study (PRA vs UKallV in NS), PRA was elevated in 8 (mean $\pm$ SEM 12.1 $\pm$ 3.7 ng/ml/h, normals 1.45 $\pm$ 0.22 ng/ml/h) and normal in 8 (1.2 $\pm$ 0.3 ng/ml/h) (Fig 30). The normal renin subjects were significantly older than the high-renin group (58.4 $\pm$ 6.2 years vs 33.8 $\pm$ 5.3 years, $p<0.05$). Differences between

Name	Plasma kallikrein (nkat/l)	Urine kallikrein (nkat/l)	Plasma/urine ratio (%)
RA	1.5	22.1	6.6
JS	8.2	31.1	26.3
PN	4.4	39	11.2
RL	4.7	30.2	15.5
AS	7.3	26.3	27.8
Mean±SD	5.2±2.6	29.7±6.3	17.5±9.3

Table 11 Plasma and urine glandular kallikrein concentrations (calculated from activity against S2266) in 5 patients with nephrotic syndrome.

these groups in creatinine clearance, urinary protein excretion, plasma albumin and urinary sodium excretion were not significant, although both groups differed from the healthy controls in all these variables, as shown in Table 9. Patients with NS in both high- and normal-renin groups were in positive sodium balance during the study (urinary sodium excretion 43.4 $\pm$ 12.7 and 51.3 $\pm$ 5.2 mmol/day) and all nephrotic subjects gained body weight during the study. In the high renin group, mean UKallV was markedly increased (40.4 $\pm$ 5.1 nkat/24 h, normals 12.0 $\pm$ 1.1) (Fig 30). Plasma volume was reduced in the three patients in this group in whom it was measured (Fig 31). In the normal renin group, mean UKallV was elevated (25.7 $\pm$ 2.4 nkat/24 h) but less so than in the high-renin patients ($p < 0.05$). Plasma volume was measured in 5 of these patients; it was normal in 3 cases and reduced in 2 (Fig 31). In the 16 patients with NS, UKallV correlated with plasma renin activity (r 0.53, $p < 0.05$), with urinary protein excretion (r 0.78, $p < 0.01$), and with urinary potassium excretion (r 0.53, $p < 0.05$). Correlations between UKallV and urinary sodium excretion, urine volume, plasma potassium and creatinine clearance were not significant ($p > 0.05$).

When the ratio of PRA to UKallV was calculated, the ratio correlated with urinary sodium excretion (r -0.53, p 0.035). Neither PRA (r -0.218) nor UKallV (r -0.088) alone correlated with UNaV, and the PRA/UKallV ratio did not correlate significantly with any other measured variable.

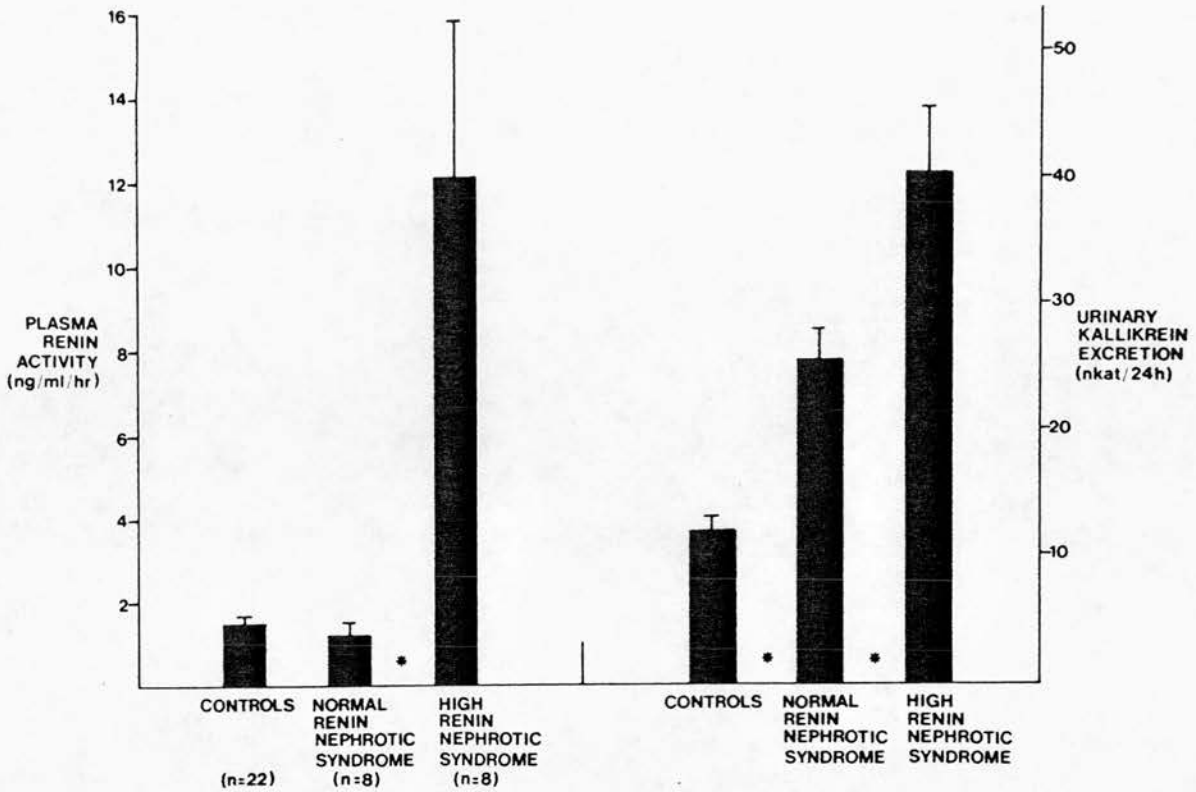


Figure 30. PRA and kallikrein excretion in nephrotic syndrome.

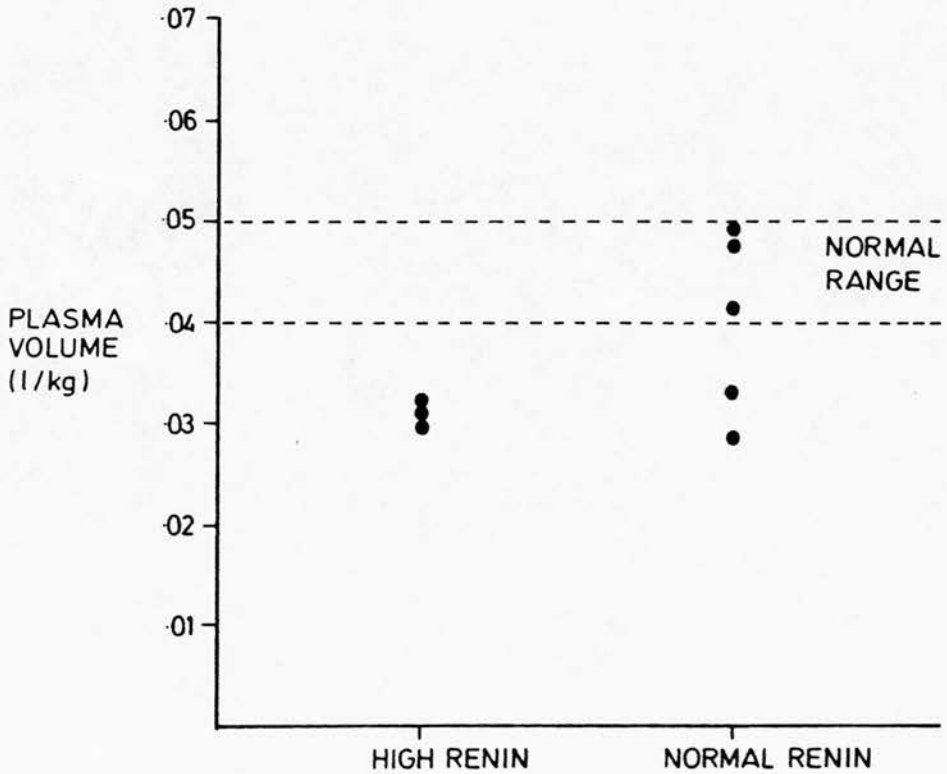


Figure 31. Plasma volumes in high- and low -renin nephrotic syndrome.

Fig 32 shows the activity of plasma against S2266 in healthy control subjects, and in patients with glomerulonephritis, with and without nephrotic syndrome. Activity in controls was low or undetectable (absorbance 1.7 x 10-3 $\pm$ 0.45 x 10-3) and similar results were obtained in glomerulonephritis (1.47 x 10-3 $\pm$ 0.36 x 10-3). Significant activity was observed in 8 of 10 nephrotic subjects (8.3 x 10-3 $\pm$ 2.1 x 10-3); this value was significantly higher than those obtained in the other groups ($p < 0.05$). In the glomerulonephritis and nephrotic groups, there was a significant overall correlation between plasma activity against S2266 and urinary protein excretion (r 0.44, $p < 0.05$).

7.4. Discussion

The reduction in UKallV in GN wihout NS probably reflects the presence of renal impairment in these patients, as discussed in Chapter 6. In contrast, patients with NS excrete supranormal amounts of urinary kallikrein, despite renal impairment in many cases. It seems unlikely that filtration from plasma can explain this finding. Plasma kallikrein has only 4% of the activity of glandular kallikrein against the substrate used in this assay (Amundsen et al, 1979). Plasma from the nephrotic patients contained only 20% of the activity against the substrate compared to undiluted urine. This value could reflect the presence in plasma of non-kallikrein proteolytic enzymes with some activity against the substrate, or the presence of

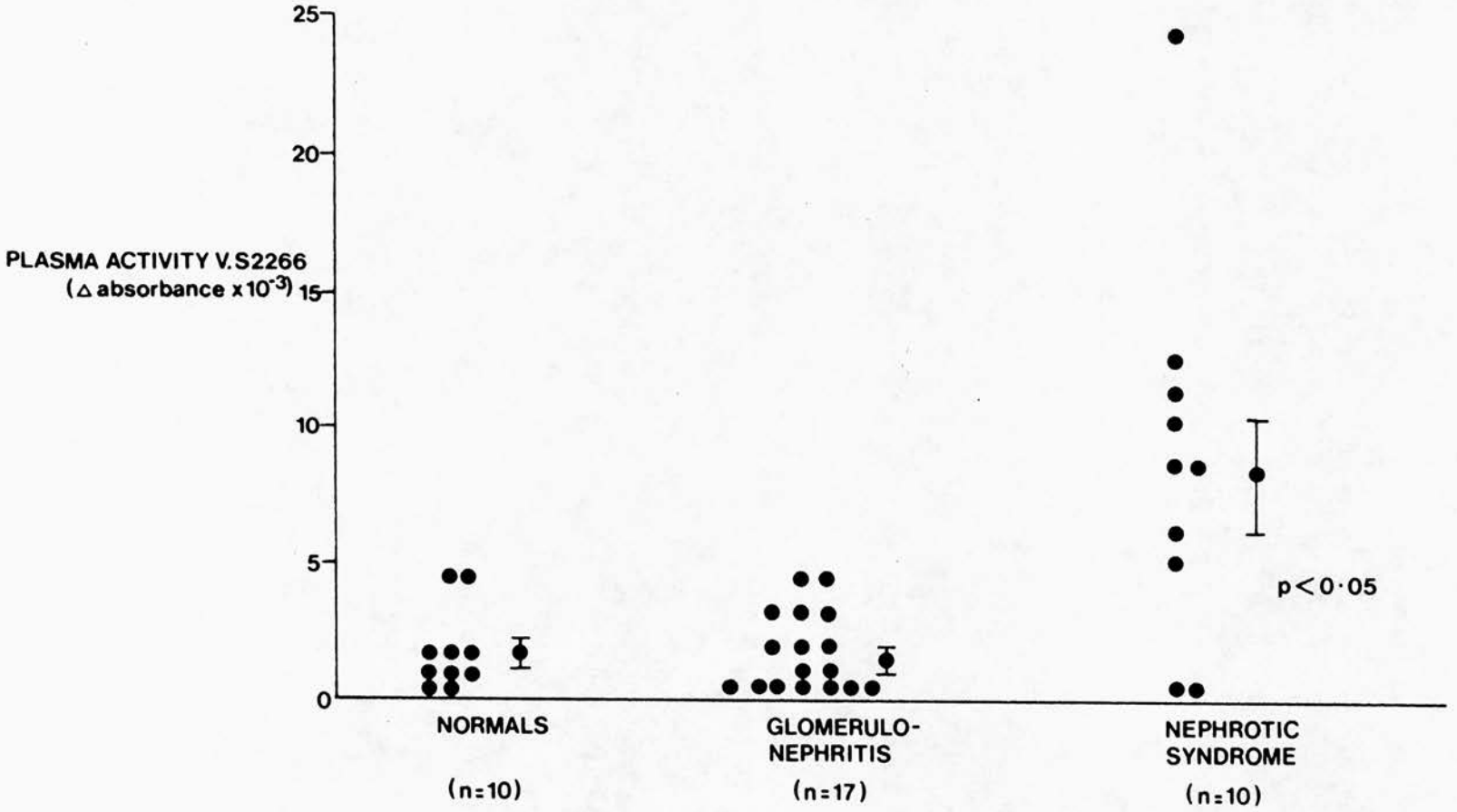


Figure 32. Activity of plasma against the glandular kallikrein substrate, S2266

glandular kallikrein in plasma , as discussed below (Rabito et al, 1983). When labelled glandular kallikrein is injected intravenously, less than 10% appears intact in urine (Mills, Paterson and Ward, 1975; Rabito et al, 1985). Glandular kallikrein would therefore have to cleared from the circulation at approximately 70 ml/min to explain the urinary kallikrein findings on the basis of filtration from plasma, which seems inconceivable for a protein of molecular weight 40,000. It therefore seems likely that kallikrein is released by the kidney into the urine in NS. We found that kallikrein activity in normal urine was unaffected by the addition of a protein solution; this suggests that kallikrein does not bind actively to protein, and that the elevated UKallV in NS is not simply due to proteinuria. Urinary kallikrein frequently increases in association with diuresis and natriuresis, and passive washout of tubular enzyme has been postulated (Seeber, Vila and Catanzaro, 1982). However, the NS patients in this study had lower urine volumes and urinary sodum excretion than the other patient groups. The explanation for increased UKallV in NS is therefore not immediately apparent.

During sodium restriction or hypovolaemic haemorrhage, plasma renin activity and urinary kallikrein activity change in parallel (Levy et al, 1977; Maier and Binder, 1979). This has been interpreted as indicating a functional link between the renin-angiotensin-aldosterone systems and the renal kallikrein-kinin system, whereby the vasodilator properties of kinins counteract the vasoconstriction due to

locally generated angiotensin II in the kidney, as previously discussed. Our finding of increased UKallV in NS could therefore have reflected activation of the renin-angiotensin-aldosterone system resulting from hypovolaemia. In the second part of this study, however, we confirmed the findings of other workers that at least 50% of patients with NS have normal PRA and plasma volumes (Meltzer et al. 1979). Despite this, they show a significant increase in UKallV compared with normal subjects. The increased UKallV in NS is, therefore, not solely a reflection of either hypovolaemia or hyperreninaemia, although UKallV was significantly greater in the high-renin group, suggesting a partial link between the two systems. The stimulus to kallikrein excretion in the normal-renin group is not clear. These patients have presumably compensated for a reduced plasma oncotic pressure by retaining sufficient sodium to expand their extracellular fluid volume (ECF) massively (Glassock, 1980). Kallikrein excretion is known to increase during acute expansion of the ECF, as previously discussed. It may be, therefore, that the marked expansion of ECF volume drives renal kallikrein release either directly or via other neural/humoral mediators. In some patients, UKallV has fallen to normal following effective diuretic therapy, but this has not been uniformly seen, (AD Cumming, unpublished observations) and the known stimulating effect of most diuretics on the renal kallikrein-kinin system makes interpretation of these findings difficult (Bonner et al,

1982; Mackay et al, 1985). Studies of kallikrein excretion in other oedematous states would be of interest in this regard; patients with alcoholic cirrhosis of the liver excrete less urinary kallikrein than normal, despite dietary sodium restriction and severe oedema and ascites (21). The possibility exists that hypoproteinaemia per se could influence kallikrein excretion, perhaps by an effect on delivery of sodium to the distal nephron (Green, Windhager and Geibisch, 1974; Grausz, Leiberman and Earley, 1972). However, as discussed in Chapter 10, an acute reduction in plasma protein concentration induced in sheep by extracorporeal plasmapheresis, does not alter urinary kallikrein excretion.

Recent studies have indicated a relationship between dietary potassium intake and urinary kallikrein excretion in rats (Vio and Figueroa, 1987), although the underlying mechanism is unclear. In this study, kallikrein excretion was positively correlated with potassium excretion, but not with excretion of sodium or water. This could indicate a link with mineralocorticoid activity, as previously suggested (Geller et al, 1972), although in the first part of this study it was demonstrated that the increased kallikrein excretion in NS was seen in patients with normal plasma aldosterone concentrations, and could not be explained purely on the basis of secondary aldosteronism. Other compounds with mineralocorticoid activity could, however, be important in this respect. The renal kallikrein-kinin system interacts in a complex fashion with other vasoactive hormone

systems, including the sympathetic nervous system, dopamine, prostaglandins, vasopressin, and atrial natriuretic peptide (Tulassay et al, 1987). Assessment of these mechanisms in parallel with kallikrein studies in nephrotic syndrome, combined where appropriate with the use of pharmacological inhibitors, may clarify the cause of the increased kallikrein excretion in NS.

Glandular kallikreins are known to be released into the systemic circulation from submandibular glands, pancreas and kidney (De Bono and Mills, 1974; Rabito et al, 1983; Scicli et al, 1983) but a role for these enzymes in physiological or pathological states has not been demonstrated, other than perhaps in acute pancreatitis (Fuller and Funder, 1987). Experimental studies in the rabbit have recently shown that when kallikrein release from the submandibular gland is stimulated and combined with kininase II inhibition, profound hypotension ensues, suggesting that under certain conditions glandular kallikreins could exert systemic effects (Rabito et al, 1983). Renal kallikrein has been identified in renal venous effluent and renal lymph (De Bono and Mills, 1974; Proud et al, 1983). Studies of renal kallikrein generation in NS are lacking. It seems likely, however, that the increased plasma activity against the glandular kallikrein substrate, S2266, seen in NS reflects release of renal kallikrein, since the urine in NS also shows very high activity against the same substrate, and exceeds the plasma activity five-

fold. The ability of this enzyme to cause systemic effects in NS would depend on the rate of release, the rate of degradation (which occurs primarily in the kidney) and the efficiency of the kininase system. The reported occurrence of proteinuria and NS in patients receiving inhibitors of angiotensin converting enzyme, which is identical to kininase II, is therefore of some interest (Atkinson and Robertson, 1979). Infusion of glandular kallikrein into the renal artery of dogs causes proteinuria, which can be abolished by the kallikrein inhibitor, aprotonin (Murakami, Hori and Masamura, 1968). Intraperitoneal injection of renal homogenates into nephrectomised rats produces a syndrome resembling nephrosis, and it has been suggested that this might be due to a factor of renal origin which increases capillary permeability - possibly kallikrein (Asscher and Anson, 1963). Increased glomerular synthesis of thromboxane has been suggested as a cause of proteinuria in nephrosis (Remuzzi et al, 1985); bradykinin and related kinins are potent stimulators of phospholipase A2, and promote synthesis of arachidonic acid metabolites, including thromboxane A2 (Regoli and Barabe, 1980). The localisation of kallikrein within the granular peripolar cell, at the vascular pole of the glomerulus, as described in Chapter 5, facilitates a role for kallikrein in glomerular pathology, including a possible effect on glomerular capillary permeability. These observations would all be compatible with participation of the renal kallikrein-kinin system in the pathogenesis of NS. Pharmacological inhibitors of both

kallikreins and kinins are available (Chapter 1) and a single uncomfirmed report suggests that they may be of value in the treatment of this frequently refractory condition (Murakami, Hori and Masamura, 1968).

The significance of the correlation between the PRA/UKallV ratio and urinary sodium excretion in NS is discussed fully in Chapter 11. As mentioned above, the reason for sodium retention in NS is unclear in many cases, and has been ascribed to an intra-renal defect in sodium handling (Firth, Raine and Ledingham, 1989). The finding of a positive correlation between the activities of these two intra-renal hormone mechanisms and sodium excretion in NS is therefore of considerable interest, although the degree of correlation is not particularly strong, suggesting that other factors are involved in what is likely to be a multi-factorial phenomenon.

In summary, our results provide evidence for disordered activity of the renal kallikrein-kinin system in NS. The increased urinary kallikrein excretion is partially, but not wholly linked to increased activity of the renin-angiotensin system. Kallikrein generated by the kidney, in addition to appearing in the urine, may reach the systemic circulation. Alterations in the relationship between the renin-angiotensin and kallikrein-kinin systems may be involved in the sodium retention of NS.

CHAPTER 8 THE PLASMA AND RENAL KALLIKREIN-KININ SYSTEMS IN HEPATIC CIRRHOSIS

8.1. Introduction

The cause of renal retention of sodium in patients with hepatic cirrhosis and ascites is not known, and a number of factors are likely to be involved (Epstein,1983). As in septic shock, the renal functional pattern resembles a "pre-renal" state, with oliguria, negative free water clearance, and reduced absolute and fractional sodium excretion. Provided the patient has not developed the "hepatorenal syndrome", GFR and renal plasma flow are, however, not markedly reduced; GFR is reduced in some patients but does not relate to the degree of sodium retention (Chaimovitz et al, 1972). Most studies have found that measured plasma volume is normal or even increased (Tristan & Cohn, 1967). The concept of a reduced "effective plasma volume" in face of a low systemic vascular resistance has been invoked, although measures to increase central volume, such as water immersion, do not normalise sodium excretion (Bichet, Groves & Schrier, 1983). Other possibilities for the "afferent" signal to the kidney include a hepatic mechanoreceptor responding to increased intra-sinusoidal pressure, which would stimulate renal sodium retention via a neural reflex arc and lead to ascites by an "overspill" mechanism of overall ECF expansion (Leiberman, Denison & Reynolds, 1970). It is possible that both these hypotheses are correct.

The "efferent" mechanism promoting renal tubular sodium

reabsorption in cirrhosis and ascites is also controversial. There is evidence of increased efferent renal sympathetic nerve activity (ERNSA), which could directly influence tubular function, although direct confirmation of this in man is lacking (Di Bona, 1984). Attention has also focused on other neuro-endocrine mechanisms, including the renin-angiotensin system, aldosterone, AVP, prostaglandins, thromboxanes, prolactin, and ANP (Epstein 1983; Better & Schrier, 1983). The possibility of a "defect" in the kallikrein-kinin system in cirrhosis was raised by Colman et al, who described a reduction in circulating prekallikrein in cirrhotic patients (Wong, Talamo & Williams, 1977). It seems likely, however, that this is due in part to reduced hepatic synthesis, rather than activation; there is also very little evidence to support a role for plasma kinins in the regulation of renal function. Zipser described an "parodoxical" reduction in urinary kallikrein excretion in cirrhosis; PRA, aldosterone, and urinary PGE2 excretion were increased in those patients with ascites, but their subjects were taking a 10 mmol sodium diet, which in itself will stimulate the renin-angiotensin-aldosterone and prostaglandin systems (Zipser, 1981). It has been recommended that, for maximum clinical relevance, studies of sodium-retaining syndromes should be conducted under conditions of positive sodium balance (Brown et al, 1982). It was therefore of interest to study the activity of the plasma and renal kallikrein-kinin systems in cirrhotic patients, with and without ascites, on an 100 mmol sodium

diet, during sodium accumulation.

8.2. Patients and methods

12 patients with established cirrhosis were studied. Six had ascites, defined as the unequivocal presence of free intraperitoneal fluid detected on clinical examination; they were studied prior to the initiation of diuretic therapy. Six did not have ascites and had never had diuretic therapy. Age and sex were similar in each group. All patients were studied in hospital, after at least 48 hours on a 100 mmol sodium diet and unrestricted oral fluids, and on no medication. The study consisted of -

1. 24 hour urine collection for volume, sodium, potassium, and kallikrein.
2. After an overnight fast, measurement of PAH and inulin clearance, as described for the control periods of the saline infusion study (Chapter 3).
3. After 30 mins supine, at 9 am, blood sampling for PRA, Ang II, aldosterone, prekallikrein, catecholamines, ANP, AVP.
4. Urine sampling during clearance study for sodium, potassium, osmols, kallikrein.

PRA, Ang II, aldosterone, ANP, and AVP were measured by established radioimmunoassays. Plasma catecholamines were measured by HPLC using electrochemical detection.

8.3. Results

Urine volume, urinary sodium excretion, and FENa were all

significantly lower in the ascitic group than the non-ascitic patients (Table 12). GFR, measured as inulin clearance, and renal plasma flow, measured as PAH clearance, were identical between the two groups, although there was considerable variation within groups (Table 12). Urinary kallikrein excretion was very low in 2 of the 6 ascitic patients, and also in two of the 6 non-ascitic patients, so that the difference between groups was not significant. PRA was higher in the ascitic group, but the difference did not achieve significance. PRA was above the reference range (>2.7 ng/ml/hr) in 3 of the 6 ascitic subjects and none of the non-ascitic patients. When the PRA/UKallV ratio was calculated as before, and log-transformed because of its extreme non-parametric distribution, the ratio was significantly higher in the ascitic patients; there was little overlap between ascitic and non-ascitic patients. The PRA/UKallV ratio correlated strongly with urinary sodium excretion (r -0.80, p 0.002)(Fig 33). Other correlations are shown in Table 13.

Compared with normals, cirrhotic patients had reduced plasma prekallikrein concentrations; there was no difference between ascitic and non-ascitic subjects (normals, 108$\pm$14% of pooled normal human plasma, ascitic 40.44 $\pm$ 3.82%, non-ascitic 55.78 $\pm$ 9.13%).

Plasma Angiotensin II was higher in the ascitic group, the difference being barely significant. There were no significant differences between the two groups in plasma aldosterone, plasma atrial natriuretic peptide, plasma

	ASCITIC	NO ASCITES	p
Urine volume ml/min	0.55 ± 0.10	3.41 ± 0.80	0.005
Urine sodium umol/min	54.2 ± 25.2	234.3 ± 56.2	0.015
FENa %	0.38 ± 0.14	1.66 ± 0.51	0.035
Inulin clearance ml/min	95.1 ± 12.9	115.0 ± 12.9	NS
PAH clearance ml/min	577.0 ± 110.5	572.7 ± 86.4	NS
Urine kallikrein nkat/min x 10-3	5.03 ± 2.06	5.95 ± 1.81	NS
Plasma renin ng/ml/hr	2.77 ± 0.98	0.58 ± 0.20	NS
PRA/UKallV ratio (log 10)	-.070 ± 0.325	-1.024 ± 0.177	0.025
P prekallikrein %NHP	40.44 ± 3.82	55.78 ± 9.13	NS
P aldosterone ng/100ml	19.00 ± 8.17	3.33 ± 2.85	NS
P Angiotensin II pg/ml	24.75 ± 10.29	3.38 ± 0.77	0.045
Plasma ANP pg/ml	71.23 ± 17.75	52.78 ± 13.81	NS
P noradrenaline nmol/l	5.175 ± 0.606	3.425 ± 1.019	NS
P adrenaline nmol/l	0.425 ± 0.025	0.356 ± 0.034	NS

Table 12 Renal function and neuro-endocrine regulatory factors in patients with hepatic cirrhosis with ascites (n=6) and without ascites (n=6). Mean ± SEM.

P = plasma
FENa = fractional sodium excretion
NHP = normal human plasma pool

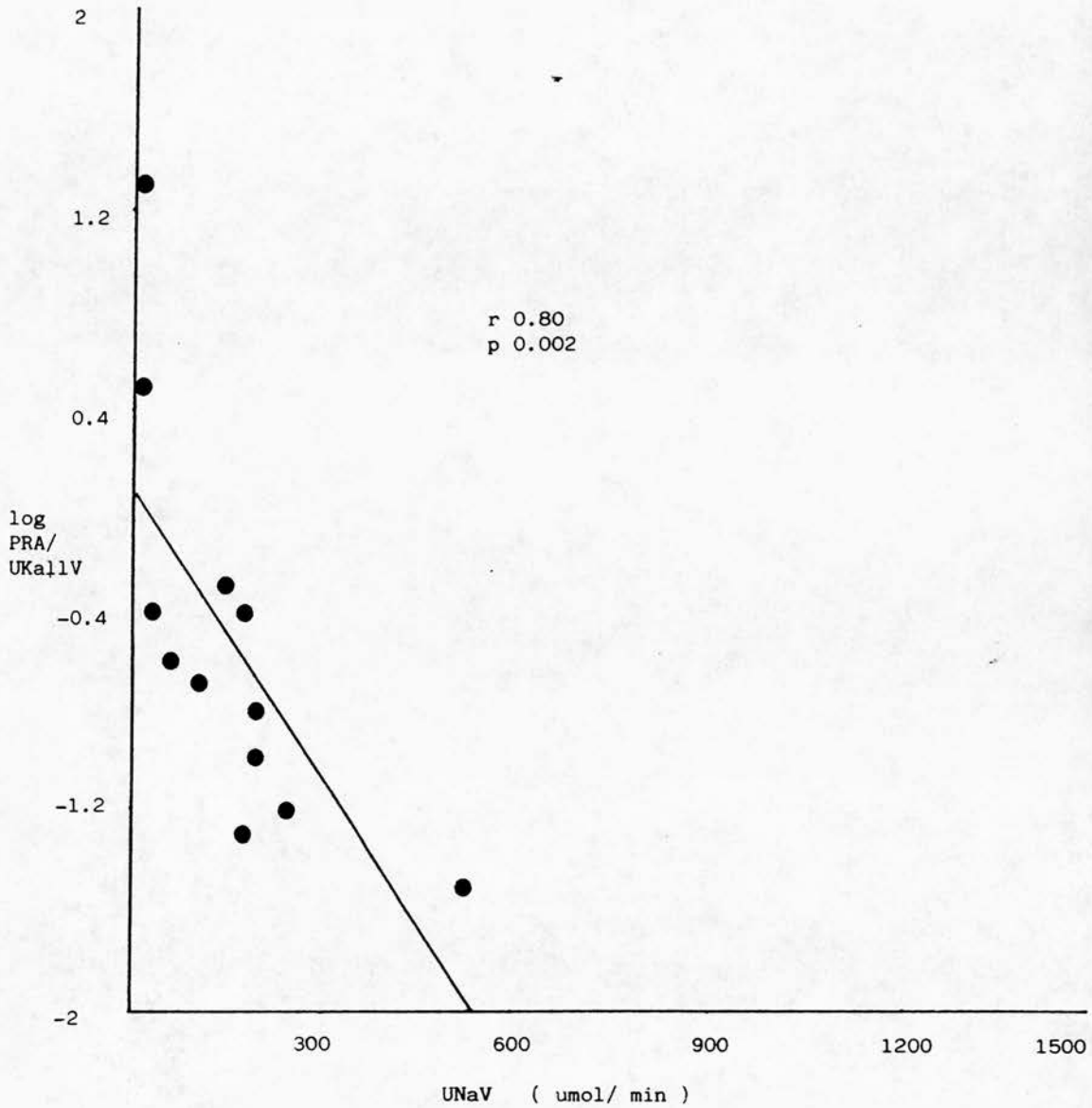


Figure 33. Correlation between log PRA/UKallV and UNaV in 12 patients with cirrhosis.

noradrenaline, or plasma adrenaline (Table 12).

In the ascitic group, plasma osmolarity was lower, urine osmolarity higher, and free water clearance lower than the non-ascitic group (Table 14).

			r	p
Vu	v	UKallV	0.54	NS
		PRA	-0.37	NS
		log PRA/UKallV	-0.73	0.007
UNaV	v	UKallV	0.50	NS
		PRA	-0.42	NS
		log PRA/UKallV	-0.80	0.002

Table 13 Correlations between urine volume and urinary sodium excretion, and urinary kallikrein excretion and PRA, in 12 patients with cirrhosis.

	ascitic	non-ascitic
Plasma osmolarity	267 ± 12	282 ± 9 *
Urine osmolarity	741 ± 103	369 ± 29 *
Free water clearance	-1.0 ± 0.14	+0.82 ± 0.06 *

* $p<0.05$

Table 14 Indices of renal water handling in 12 patients with cirrhosis.

8.4. Discussion

As in a previous study, the plasma prekallikrein concentration was found to be reduced in cirrhosis (Wong, 1977). It is still unclear to what extent this reflects activation or reduced synthesis, but even if activation of

the plasma kallikrein-kinin system is present, it seems unlikely that a direct effect on renal function occurs. Such kallikrein-kinin activation could, however, be involved in the systemic vasodilation seen in cirrhosis, which, by reducing central "effective volume", could act as a stimulus to sodium-retaining mechanisms (Nicholls et al, 1986). Endotoxin is a potent activator of the plasma kallikrein-kinin system, and endotoxaemia is frequent in patients with advanced liver disease (Wardle, 1982). It would be of interest to study the effect of kallikrein inhibition in such patients.

The results clearly show that the profound sodium and water retention seen in cirrhosis with ascites is independent of renal haemodynamics, and represents primarily an alteration in renal tubular handling of salt and water. A number of factors are likely to be involved in this syndrome. The plasma and urinary osmolarity data are pathognomonic of increased circulating AVP in the ascitic group; plasma AVP is being measured, but the results are not available at the time of writing (Skowsky & Fichman, 1984). AVP alone cannot explain the sodium retention, however; sodium retention is not a feature of the syndrome of inappropriate AVP secretion, for example, and non-pressor AVP analogues suppress PRA and increase UNaV (Malayan & Reid, 1982). Schrier's group have emphasised the role of the sympathetic nervous system in promoting sodium retention and AVP release, and have observed increased plasma catecholamines in cirrhosis, which correlated with sodium excretion (Nicholls et al, 1986).

Epstein's group have not confirmed these results, however (Epstein, 1983). In the present study, we found no difference in plasma catecholamines between sodium retaining, ascitic subjects and the non-ascitic group; while plasma catecholamines are at best a poor index of overall sympathetic nervous system activity, the results suggest that the sympathetic nervous system is not a "prime mover" in the sodium retention.

The role of aldosterone has been emphasised (Wilkinson et al, 1979); while plasma aldosterone tended to be higher in the ascitic group, the difference was not significant at the 5% level. Similarly, Epstein's group have studied cirrhotic subjects under rigidly controlled conditions, off diuretics, and found elevated plasma aldosterone in only 4 of 28 patients (Epstein, 1983). A renin-angiotensin effect is possible, and is supported by the trend to higher PRA and significant elevation of plasma Angiotensin II concentration in the ascitic group. A pure tubular action of AII would be unusual, however (Kotchen, 1983). PRA was not significantly elevated in the ascitic patients, and did not correlate with sodium or water excretion overall (Table 13). There is no evidence that blockade of the renin-angiotensin system is beneficial in cirrhosis (Schroeder et al, 1976), and hypotension frequently results. We found no difference in UKallV between ascitic and non-ascitic subjects; while some patients in each group had very low values, overall the results were exactly comparable to basal values in normal

subjects (Figures 9 & 15). UKallV alone did not correlate with water or sodium excretion (Table 13). We have therefore not confirmed the findings of Zipser et al (1981) and of Perez-Ayuso et al (1984), of reduced urinary kallikrein excretion in cirrhosis. Both these groups used a less specific kallikrein assay, and their results may have been influenced by a very low sodium diet. Therefore, neither PRA nor UKallV alone stand as determinants of sodium handling in our study. However, the degree of correlation between the PRA/UKallV ratio in this study is such that approximately 65% of the variability in sodium excretion can be ascribed to changes in this ratio. No other variable, to my knowledge, has correlated with UNaV to this extent in previous studies. Comparison of the regression line with those seen in normal subjects (Chapter 3) indicates a similar curve overall; the ascitic patients lie to the left of the curve (high ratio, low UNaV). This can be interpreted as indicating that the PRA/UKallV - UNaV relationship is influenced in the direction of anti-natriuresis in ascitic subjects. Further discussion of the factors which could effect this change is contained in Chapter 11.

CHAPTER 9 THE RENAL KALLIKREIN-KININ SYSTEM AND OTHER VASOACTIVE HORMONE SYSTEMS IN THE RENAL RESPONSE TO NON-HYPOTENSIVE SEPSIS

9.1. Introduction

Sepsis and acute renal failure are frequently associated (Werb and Linton, 1979), and up to 50% of cases of acute renal failure are due to septicaemia (Beaman et al, 1987). The pathogenesis of this syndrome is poorly understood. The importance of endotoxin in mediating renal damage has been emphasized (Wardle, 1982); experimental endotoxaemia is ordinarily associated with hypotension, systemic and renal vasoconstriction, and intrarenal coagulation with glomerular thrombosis (Balis et al, 1978; Conger, Falk and Guggenheim, 1981). However, both clinical and experimental observations indicate that acute renal failure may supervene in sepsis in the absence of hypotension and structural renal damage (Richmond et al, 1985; Walker et al, 1986). It has been suggested that this is a type of "functional" renal failure associated with reduced glomerular filtration and sodium retention, despite adequate renal perfusion (Walker et al, 1986); it may progress to established acute tubular necrosis, but initially may be amenable to appropriate therapy. The renal kallikrein-kinin system and other neuro-endocrine mechanisms known to influence renal function could be involved in the pathogenesis of this form of acute renal failure; this possibility was investigated in a large animal (ovine) model of intra-peritoneal and systemic sepsis.

9.2. Materials and Methods

The technique of surgical induction of peritonitis was modified from that of Wichterman (Wichterman, Baur and Chaudry, 1980). Sixteen healthy sheep aged 12-18 months and weighing 40-50 kg underwent general anaesthesia and cannulation of the common carotid artery, and the pulmonary artery via the external jugular vein with a triple-lumen Swan Ganz catheter. The bladder was catheterized per urethra. After recovery from anaesthesia, animals were housed in metabolic cages and volume-loaded with 8 litres of Ringer's lactate intravenously (IV) over 24 hours. After control haemodynamic measurements and blood and urine sampling, animals underwent a second general anaesthetic; peritonitis was induced by devascularization and proximal ligation of 6-8 cm of distal caecum, combined with partial omentectomy and a 2 cm puncture in the caecal tip. The abdomen was closed and the animal allowed to recover from anaesthesia. Post-operatively all animals received 50 mg pethidine and 25 mg chlorpromazine IV and were continued thereafter on an IV infusion of pethidine, 50 mg/6 hours. IV infusion of Ringer's solution, 8 litres/24 hours, continued for the duration of the experiment. Follow-up haemodynamic measurements and blood and urine collections were made 24 hours after induction of sepsis. Animals were supervised continuously during the study. Any animal seen to be restless, agitated or showing other evidence of discomfort or distress despite pethidine infusion was sacrificed immediately by IV injection of pentobarbital

(Euthanol). Animals surviving through 24 hours were sacrificed as above; an open renal biopsy was taken at the time of death.

Haemodynamic parameters measured were mean arterial pressure (MAP), central venous pressure (CVP), mean pulmonary artery pressure (PAP), pulmonary capillary wedge pressure (PCWP), and thermodilution cardiac output. Cardiac index (CI) and systemic vascular resistance index (SVRI) were derived by standard formulae. Blood samples were taken from the aortic cannula; white cell count, serum creatinine, sodium and albumin were measured by standard autoanalyzer techniques. Plasma renin activity (PRA) was measured by radioimmunoassay (Haber et al, 1969). Arterial plasma norepinephrine and epinephrine were measured by high-performance liquid chromatography and electrochemical detection (Bioanalytical Systems Inc., 1985). The lower limit of detection in this assay was 100 pg/ml; some samples showed undetectable plasma epinephrine and were arbitrarily allocated a value of 100 pg/ml for statistical purposes. Urine was collected for 4 hours on the morning of each study day and the volume recorded; creatinine and sodium were measured, and urinary kallikrein concentration assayed as previously. The urinary concentration of 6-keto PGF 1α and thromboxane B2 were measured by radioimmunoassay (Ali and McDonald, 1980) and urinary excretion rates calculated. Effective renal plasma flow was measured as the clearance of orthoiodinated hippuran labelled with I128 (Dubovsky and Russel, 1982).

Clearance from plasma of technetium-chelated diethylenetriamine pentaacetic acid (99m-Tc-DTPA) which is handled by the kidney in a manner similar to inulin, was measured as an additional indicator of glomerular filtration rate (Bianchi, 1979). In each case, the isotope-labelled compound was injected via the venous catheter and all blood samples taken from the aortic cannula. Kidney tissue was examined by light microscopy using standard methods (Solez et al, 1980).

For each variable, within-group comparisons between results at baseline (pre-sepsis) and after 24 hours of sepsis and between-group comparisons for each variable at baseline and at 24 hours were made by Student-t test and where appropriate by non-parametric tests (Wilcoxon Signed Rank or Rank Sum Tests). Results are shown as mean $\pm$ SEM.

The study conformed to the regulations regarding the care of experimental animals of the Canadian Council on Animal Care, and the protocol was approved by the hospital committee supervising care of experimental animals and the Ethics Committee of the University of Western Ontario.

9.3. Results

As in the studies performed to establish and validate this experimental model (Richmond et al, 1986), all 16 animals had a polymicrobial peritonitis and bacteremia after 24 hours. Organisms grown on blood culture included E.Coli, Serratia, Enterobacter, Pseudomonas and Bacteroides species; autopsy showed generalised purulent peritonitis and an

inflammatory mass in the right lower quadrant. Eight of the 16 animals studied developed acute renal failure, as shown by an increase in serum creatinine. Since the primary interest was in mechanisms of acute renal failure, the animals were divided retrospectively into those with an increase in serum creatinine (Group 1, n = 8) and those without (Group 2, n = 8), for the purpose of statistical analysis. As shown in Table 15 and Figs 34-36, the increased serum creatinine in Group 1 animals was accompanied by a fall in GFR as measured by creatinine clearance and clearance of DTPA, and marked reductions in urine flow rate and both absolute and fractional excretion of sodium. This renal functional change was noted despite maintained systemic blood pressure, increased CI, reduced SVRI and maintained CVP and PCWP (Table 16), and despite only a slight and non-significant reduction in renal plasma flow (Fig 35). Group 2 animals showed a moderate reduction in urinary sodium excretion, but no change in GFR. The increase in CI and fall in SVRI did not achieve significance in Group 2; MAP, CVP, PCWP and renal plasma flow were maintained at pre-sepsis levels. In addition to the changes in CI and SVRI in Group 1, other markers of severe sepsis in these animals were an increase in PAP and a more marked fall in WBC and plasma albumin than in Group 2 (Table 16).

No consistent or striking changes in renal histology were noted either in animals developing renal failure or in those which did not. The changes which were observed included

	Group 1 (n=8)		Group 2 (n=8)	
	Baseline	24 hours	Baseline	24 hours
Serum creatinine (umol/l)	76±4	124±16**	76±6	74±5
Creatinine Clearance (ml/min)	142±15	43±8**	152±30	123±14
C DTPA (ml/min)	134±14	39±17**	152±18	140±12
ERPF(Chippuran) (ml/min)	875±96	674±75	792±19	746±120
Urine volume (ml/4h)	1219±165	178±72**	1005±260	771±246
Urinary sodium excretion (mmol/4h)	134±15	6±2**	117±18	81±31*
Fractional sodium excretion (%)	2.9±0.5	0.5±0.2**	2.6±0.5	2.0±0.7

* different from baseline ($p < 0.05$)
** different from baseline ($p < 0.01$)

Table 15. Indices of renal function at baseline and 24 hours after surgical induction of peritonitis (mean ± SEM).

ERPF = effective renal plasma flow

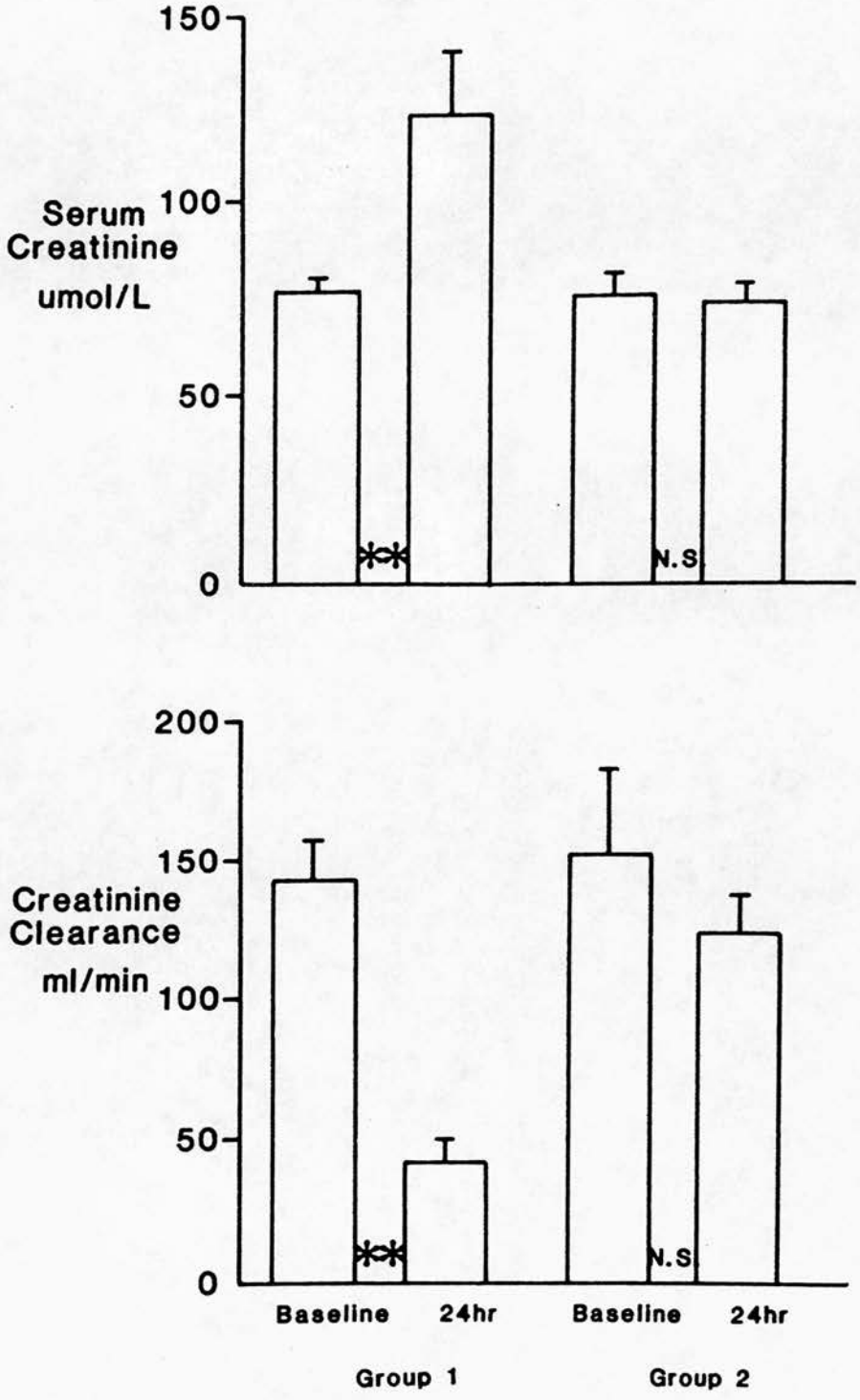


Figure 34. Serum creatinine and creatinine clearance in 16 sheep ; sepsis study.

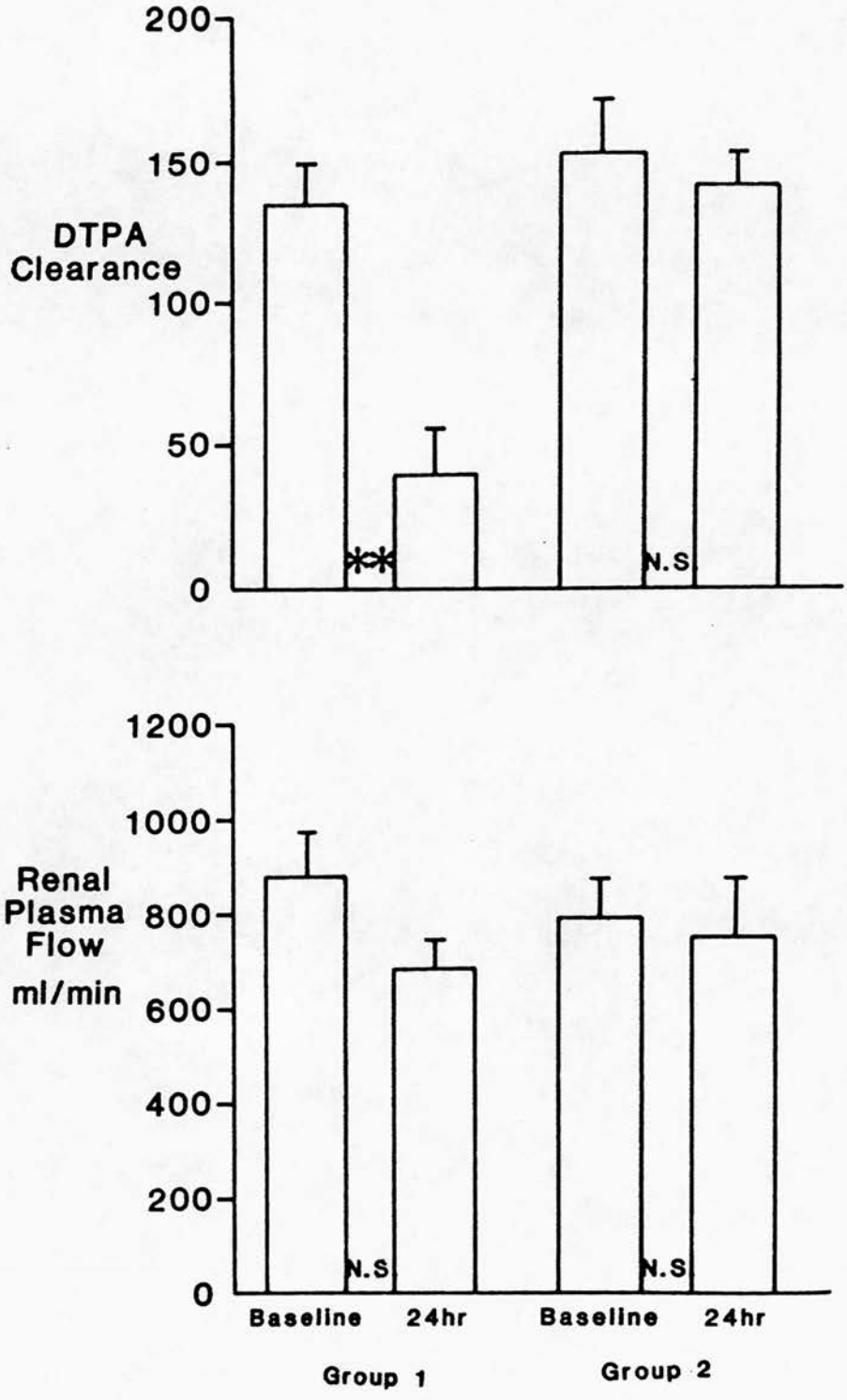


Figure 35. DTPA clearance and renal plasma flow in 16 sheep; sepsis study.

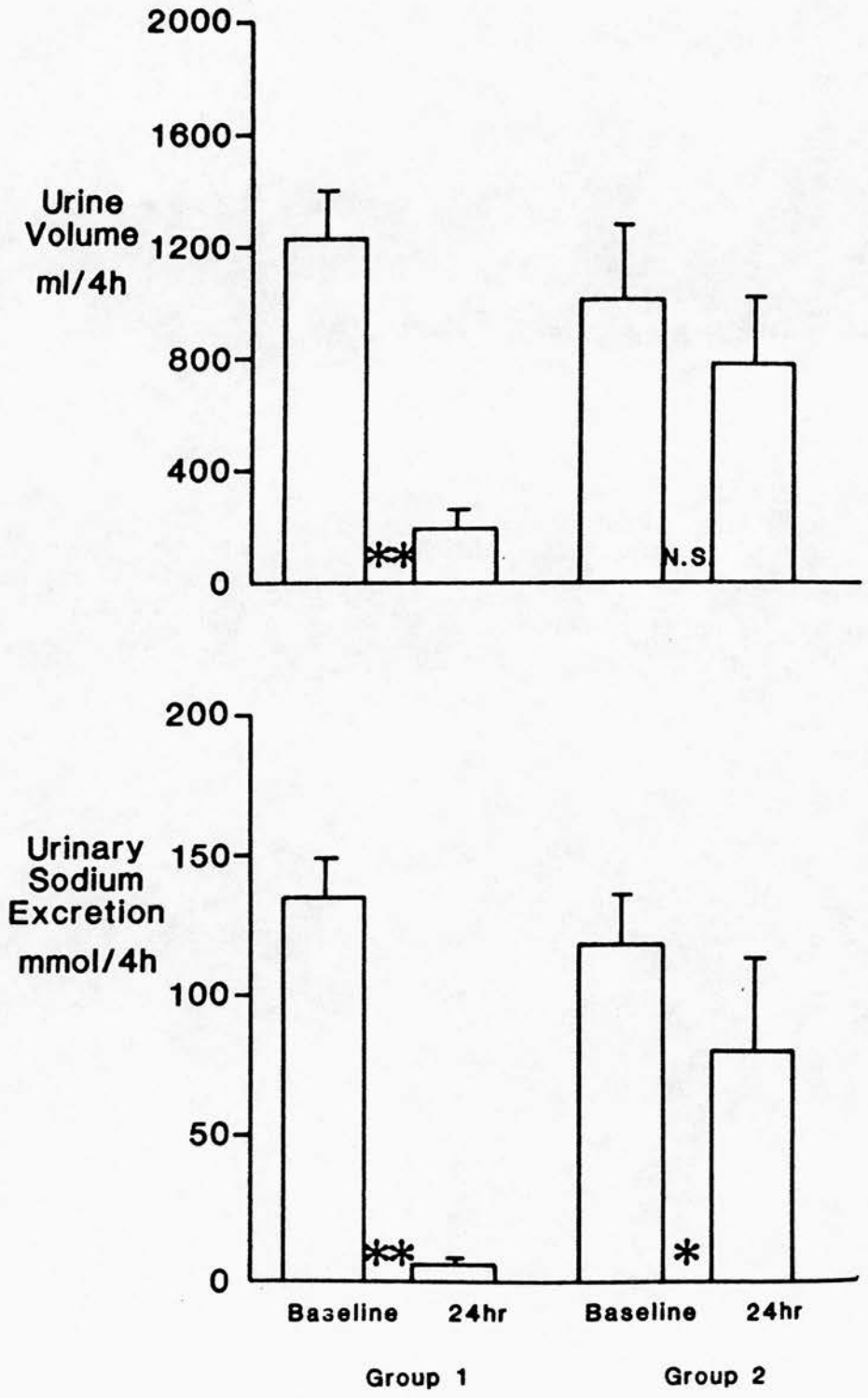


Figure 36. Urine volume and sodium excretion in 16 sheep; sepsis study.

	Group 1		Group 2	
	Baseline	24 hours	Baseline	24 hours
Mean arterial pressure (mm Hg)	108±5	90±3*	96±4	100±6
Cardiac index (L/min/m2)	4.4±0.6	7.6±0.9**	4.1±0.4	4.8±0.4
SVRI (d/sec/cm-5/m2)	2183±243	1038±107*	2026±244	1677±179
Central venous pressure (mm Hg)	2.8±1.1	4.4±0.5	3.9±1.1	4.0±1.1
PCWP (mm Hg)	9.3±0.8	10.5±1.2	9.5±1.4	10.4±1.1
Pulmonary artery pressure (mm Hg)	16±1.3	22±1.1*	18±1.8	21±1.0
White cell count (x 109/L)	8.8±1.1	2.1±0.2**	0.4±1.0	5.4±0.8
Plasma albumin (g/dl)	30.3±1.4	20.8±1.9**	32.1±1.5	27±1.1

* different from baseline ($p < 0.05$)
** different from baseline ($p < 0.01$)

Table 16. Haemodynamic variables and indicators of sepsis before and 24 hours after surgical induction of peritonitis (mean ± SEM)

SVRI = systemic vascular resistance index
PCWP = pulmonary capillary wedge pressure

hyperplasia of the juxtaglomerular apparatus (6 animals), mild tubular dilatation (8 animals), very occasional foci of tubular regeneration (4 animals), patchy interstitial infiltrate of mononuclear cells (11 animals) and small foci of medullary calcification (10 animals). These changes were evenly distributed among animals with and without renal failure.

Hormonal changes accompanying sepsis are shown in Table 17 and Fig 37. In Group 1, PRA and urinary excretion rate of 6-keto PGF1α increased after 24 hours of sepsis. Urinary kallikrein excretion was reduced compared with baseline values. Urinary TXB2 excretion rate was unchanged. In Group 2, PRA and excretion rates of 6-keto PGF1α , TXB2 and kallikrein were unchanged after 24 hours.

All animals in Group 1 showed at least a 2-fold increase in plasma norepinephrine after 24 hours of sepsis (mean $\pm$ SEM pre-sepsis, 473 $\pm$ 115 pg/ml, post sepsis 1183 $\pm$ 159 pg/ml, $p < 0.001$) (Figure 38). Plasma epinephrine in Group 1 increased in 5 animals, and the increase in mean was significant (pre-sepsis, 108 $\pm$ 8 pg/ml, post-sepsis 309 $\pm$ 70 pg/ml, $p < 0.05$). In Group 2, neither plasma norepinephrine or epinephrine increased significantly after 24 hours (Figure 38).

Studies of correlation indicated that in the 16 animals studied, there were significant correlations ($p < 0.05$) between serum creatinine and indicators of severe sepsis, namely CI (r 0.49), SVRI (r -0.55), WBC (r -0.52) and total

	Group 1		Group 2	
	Baseline	24 hours	Baseline	24 hours
Plasma renin activity (ng/ml/hr)	1.12±.48	6.48±1.86**	0.41±.06	O.62±.16
Urine kallikrein excretion (nkat/4h)	4.34±1.28	1.11±0.41*	2.05±0.23	2.08±0.51
Urine 6-keto PGF1α (ng/min)	7.6±2.	19.9±9.0*	6.2±2.7	9.6±2.8
Urine TXB2 (ng/min)	24.5±11.3	19.4±10.1	24.9±13	18.2±6.1

Table 17. Hormonal changes before and 24 hours after surgical induction of peritonitis (mean ± SEM)

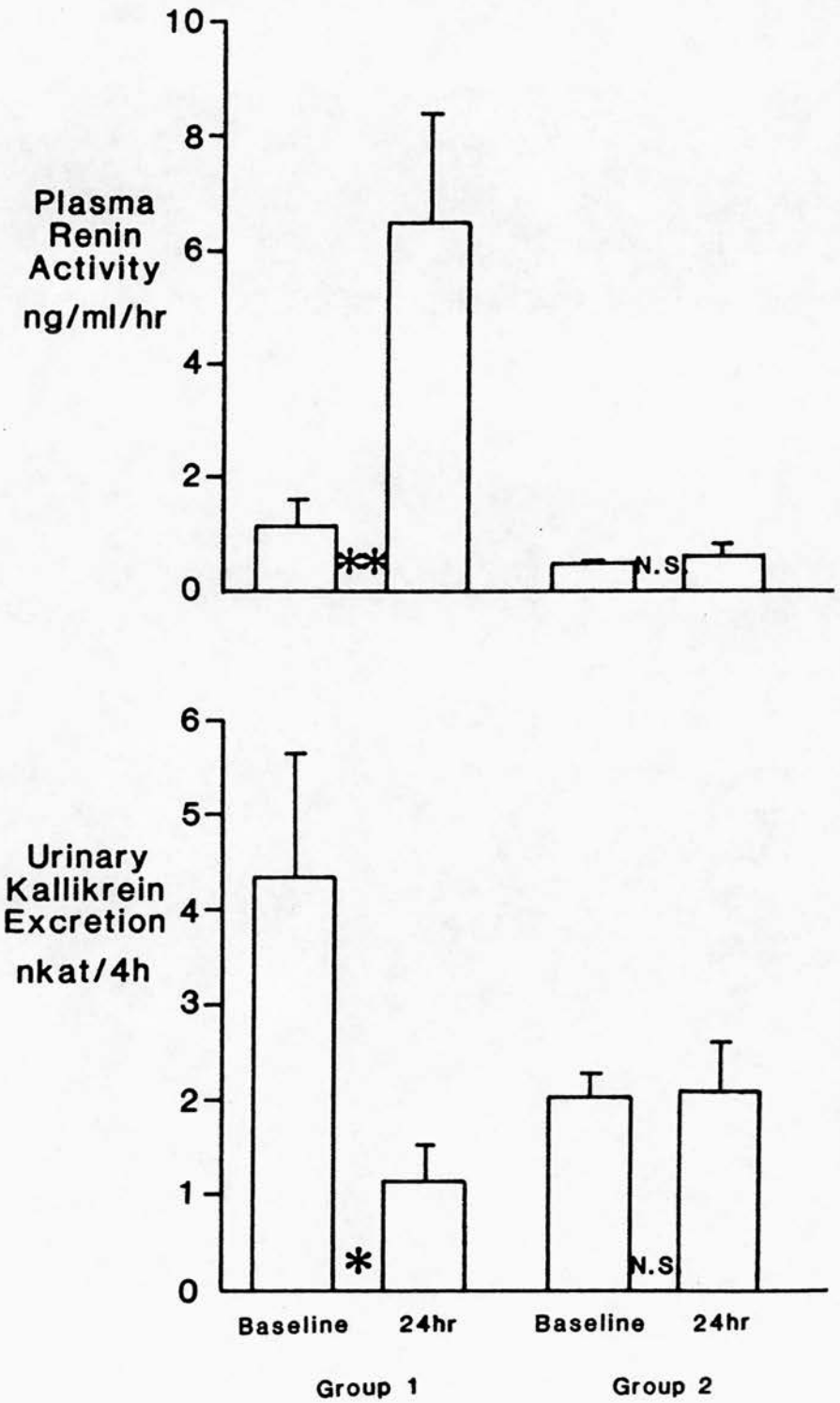


Figure 37. PRA and Urinary kallikrein excretion in 16 sheep; sepsis study.

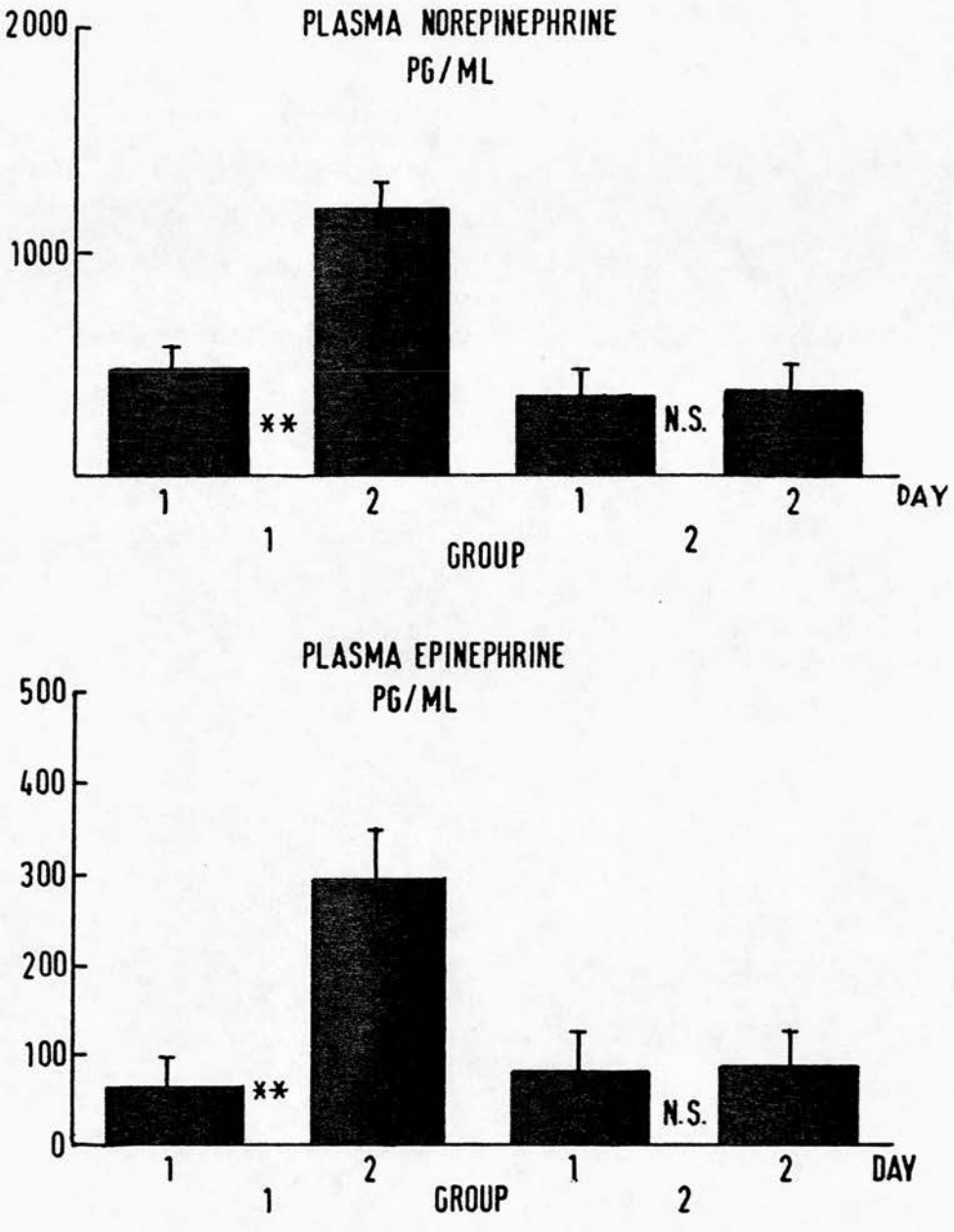


Figure 38. Plasma catecholamines in 16 sheep; sepsis study.

protein (r -0.45). Plasma renin activity correlated with serum creatinine (r 0.50) and inversely with C DTPA (r -0.52), urine volume (r -0.67), urinary sodium excretion (r -0.74) and plasma albumin (r -0.52). Urinary kallikrein excretion correlated positively with urine volume (r 0.74), urinary sodium excretion (r 0.57), and plasma albumin (r 0.75). In the renal failure group, urinary kallikrein excretion correlated with urine volume (r 0.76) and serum albumin (r 0.76). Overall, there were significant inverse correlations between plasma norepinephrine and SVRI (r -0.62), serum albumin (r -0.70), white cell count (r -0.60), and DTPA clearance (r -0.57), and a positive correlation with CI (r 0.64) and plasma epinephrine (r 0.58). Plasma epinephrine correlated with plasma renin activity (r 0.61). Combining the PRA and the urinary kallikrein excretion rate as a PRA/UKallV ratio revealed a highly significant correlation between the ratio and urinary sodium excretion (r -0.82, $p<0.01$). There were weaker ($p<0.05$) correlations between the ratio and plasma norepinephrine (r 0.55) and plasma albumin (r -0.62).

9.4. Discussion

The animal model of sepsis utilised in this study differs from most other experimental models of sepsis and endotoxemia in that it reproduces the state of volume-loaded, normotensive or near-normotensive, and vasodilated septic shock typically seen in clinical practice (Kikeri et al, 1986). Use of a large animal provides sufficient sample

size to permit simultaneous assessment of several vasoactive hormone systems in the same animal. In this model, acute renal failure reliably ensues in the presence of severe sepsis, as manifested by increased CI, decreased SVRI, increased PAP, and a large decline in white cell count and plasma albumin (Walker et al, 1986). The pattern of renal dysfunction, ie, reduced GFR, reduced urine volume and very low absolute and fractional sodium excretion, resembles that associated with reduced renal perfusion - "pre-renal" renal failure (Pru and Kjellstrand, 1985). In severe sepsis, however, it occurs despite normal systemic blood pressure, maintained CVP and PCWP, and preserved renal plasma flow. There is no evidence of severe renal structural damage, either to glomeruli or tubules, sufficient to account for the marked reduction in GFR and sodium/water excretion. This type of "sodium-retaining" acute renal failure characterised by low fractional sodium excretion, but distinct from renal hypoperfusion and intrinsic renal damage, has recently been documented in early experimental endotoxemia in rats (Kikeri et al, 1986) and in several clinical contexts, including sepsis, burns, myoglobinuria, and hepatic cirrhosis (Saha et al, 1987).

The pathophysiology underlying this syndrome remains unclear, but it may reflect at least in part the influence of systemic and intra-renal vasoactive hormone systems known to affect renal function (Schaller et al, 1985). Decreased urinary kallikrein excretion, with increased PRA and increased excretion of 6-keto PGF1α, was observed in animals

developing renal failure. The explanation for these observations, in the absence of significant hypotension or volume contraction, is not readily apparent. It is known, however, that under physiological conditions, activation of the sympathetic nervous system either by nerve stimulation or catecholamine infusion, can mediate increased renal renin release (Johnson, Schrier and Barger, 1979), decreased urinary excretion of kallikrein (Albertini et al, 1981), and can activate phospholipase enzymes leading to increased renal synthesis of prostanoids (Vandongen, O'Dwyer and Barden, 1982). It is therefore of interest that all the animals which developed renal failure during sepsis showed at least a two-fold increase in plasma norepinephrine, and the majority also showed a rise in plasma epinephrine. Statistically plasma norepinephrine correlated inversely with GFR, and also correlated with several indicators of systemic sepsis - raised CI and reduced SVRI, serum albumin and WBC. This suggests that systemic sepsis sufficiently severe to cause a hyperdynamic, vasodilated circulation is associated with increased sympathetic activity, secondary changes in intra-renal neuro-endocrine systems such as renin-angiotensin and kallikrein-kinin, and the development of renal failure. The statistical correlations could, however, be casual and do not demonstrate a functional relationship. The reciprocal changes in PRA and kallikrein excretion seen in our study resemble the effects of SNS stimulation under physiological conditions, and contrast

with the effect of other experimental procedures such as sodium depletion or hypotensive hemorrhage, which in general alter these parameters in parallel (Maier and Binder, 1979). The overall effect of the renin-angiotensin-aldosterone system on kidney function is vasoconstrictor and sodium-retaining (Kotchens and Roy, 1983), whereas the renal kallikrein-kinin system may mediate vasodilatation and facilitate sodium and water excretion, as previously discussed. These indirect effects of SNS stimulation in sepsis could therefore augment any direct effects on renal function.

As in other contexts described in previous Chapters, the strongest determinant of sodium excretion in this study was the PRA/UKallV ratio. The studies of correlation suggested an influence of the sympathetic nervous system on this parameter. They also suggested that the serum albumin concentration, which fell rapidly during sepsis, might be an important influence on these intra-renal systems. This possibility is explored further in Chapter 10.

Plasma epinephrine correlated with PRA; this is of interest since intravenous infusion of epinephrine is more effective in raising PRA than norepinephrine in conscious dogs (Johnson, Schrier and Barger, 1979). In addition, angiotensin II potentiates neuromuscular transmission and stimulates release of epinephrine from adrenal medulla (Harrison, Birbari and Seaton, 1973); this mechanism could amplify the neuro-endocrine response to sepsis.

The initial event which triggers systemic vasodilation in

sepsis, leading to the compensatory responses described above, is unclear. However, bacterial endotoxins are potent activators of the plasma "contact systems", through Hageman factor; these include the plasma kallikrein-kinin system, as outlined in the introduction (Kaplan, Meier and Mandle, 1977). It is known that the plasma kallikrein-kinin system is activated in septic shock, and kinins may well be important in the systemic vasodilation and increased capillary permeability seen in sepsis (Mason et al, 1970). We were unable to measure plasma prekallikrein in sheep because of very low activity of sheep kallikrein against S2302. Figure 39, however, shows plasma prekallikrein in 9 patients with septic shock and acute renal failure; all patients had values below the normal range, and the survivors showed a return to normal during recovery. Experimental studies of the effect of kallikrein inhibition with aprotonin in the sheep model are in progress in our laboratory. While inhibition of the renal kallikrein-kinin system might exaggerate the renal response if the above hypotheses are correct, this might be outweighed by a beneficial effect on systemic haemodynamics because of reduced plasma kinin generation. An outline of the hypothetical mechanisms leading to sodium-retaining acute renal failure during non-hypotensive sepsis is shown in Figure 40, indicating the putative involvement of the plasma and renal kallikrein-kinin systems. Clinical studies of these putative mechanisms are in progress; it is clear that

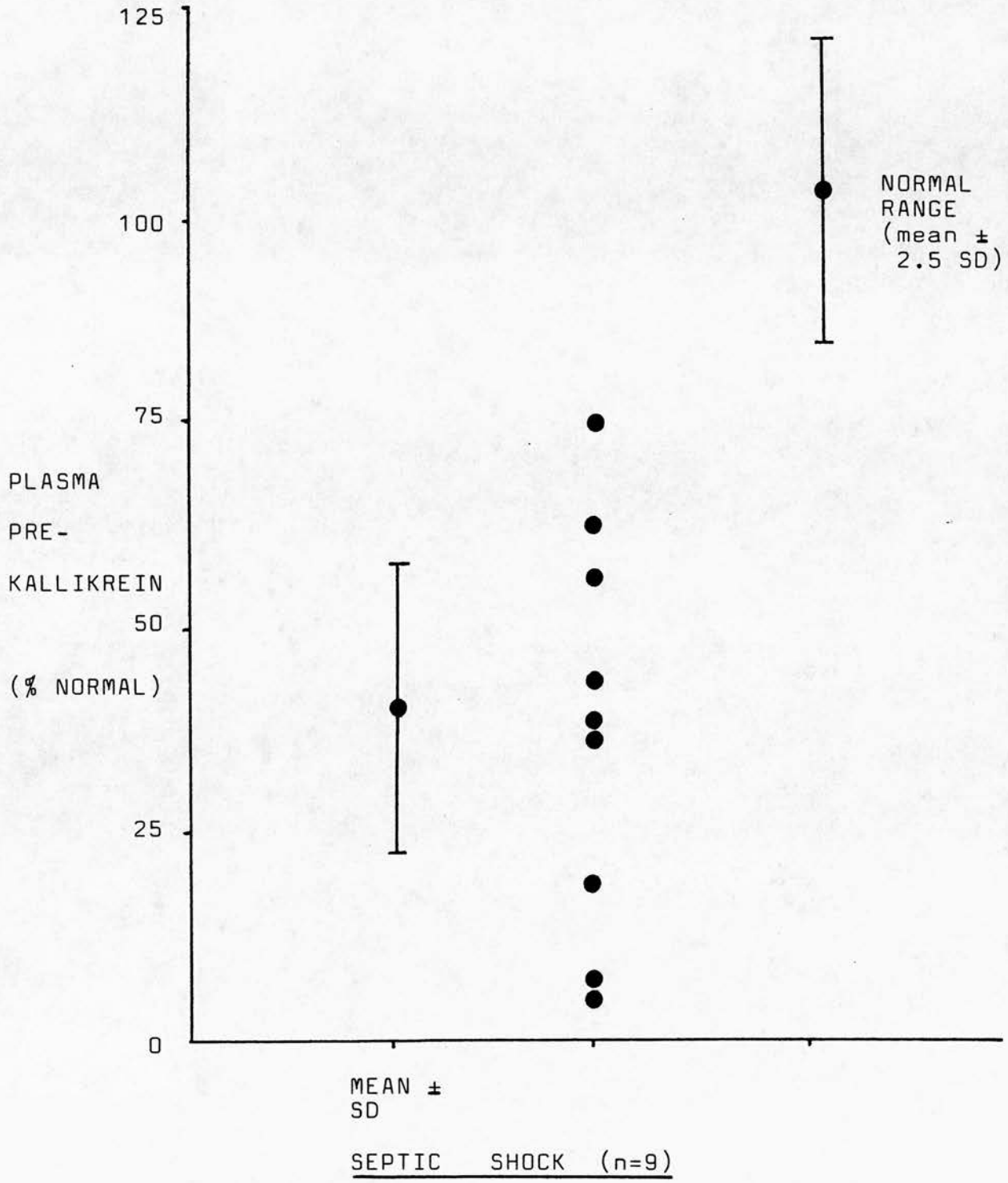


Figure 39. Plasma prekallikrein in 9 patients with septic shock.

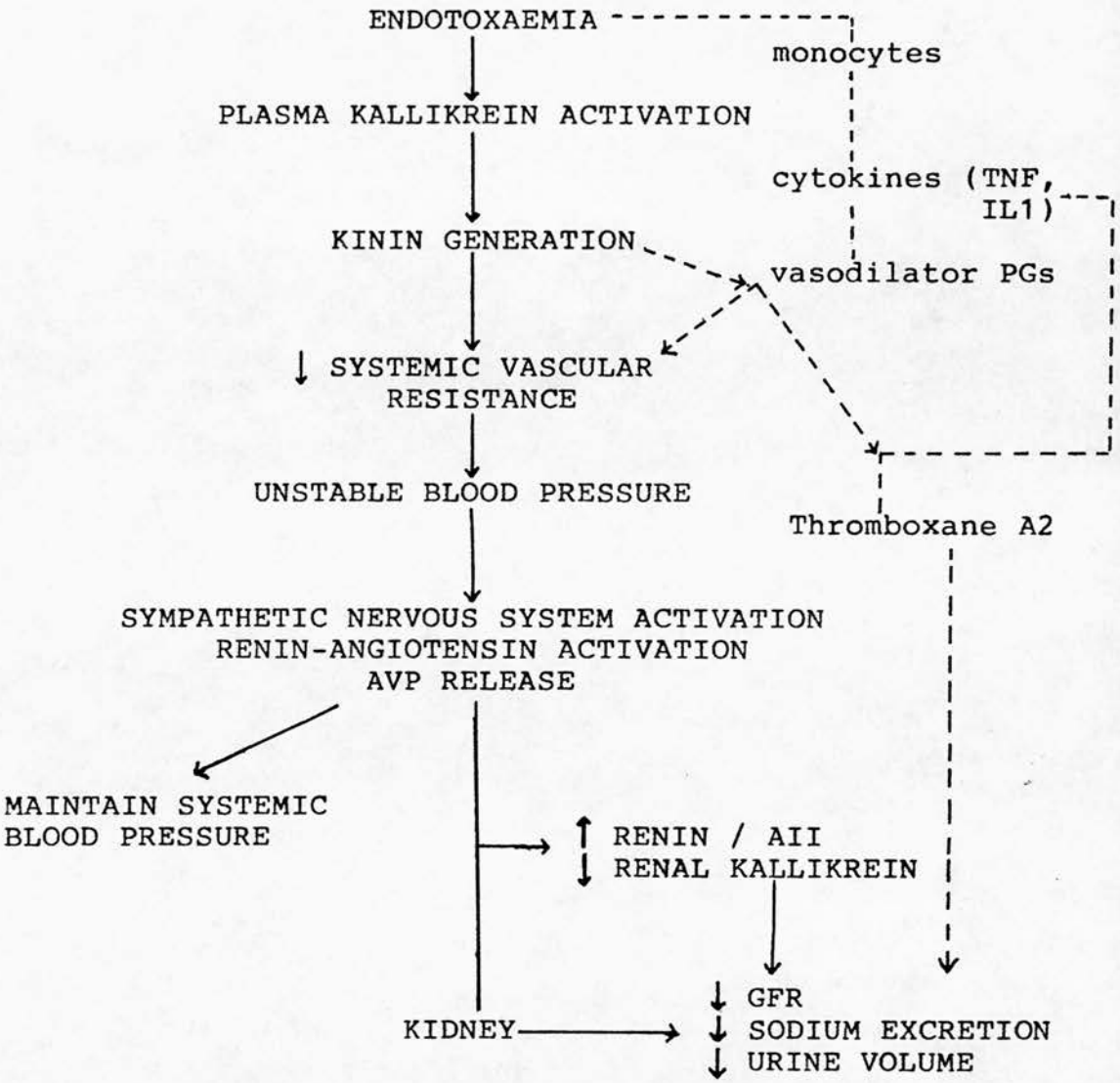


Figure 40. Outline of hypothetical mechanism for the ocurrence of "sodium-retaining" acute renal failure in sepsis, indicating possible roles of plasma and renal kallikrein-kinin systems.

"sodium-retaining" acute renal failure does occur in patients with sepsis, and that a similar pattern of renal function and of neuro-endocrine systems is seen. Figs 41 and 42 show detail of a 42-year old female with a wound infection after spinal surgery, and Serratia marcensens on blood cultures, during a period of acute renal failure and during convalescense.

FIGURE 41 ES, ♀, 42 yrs

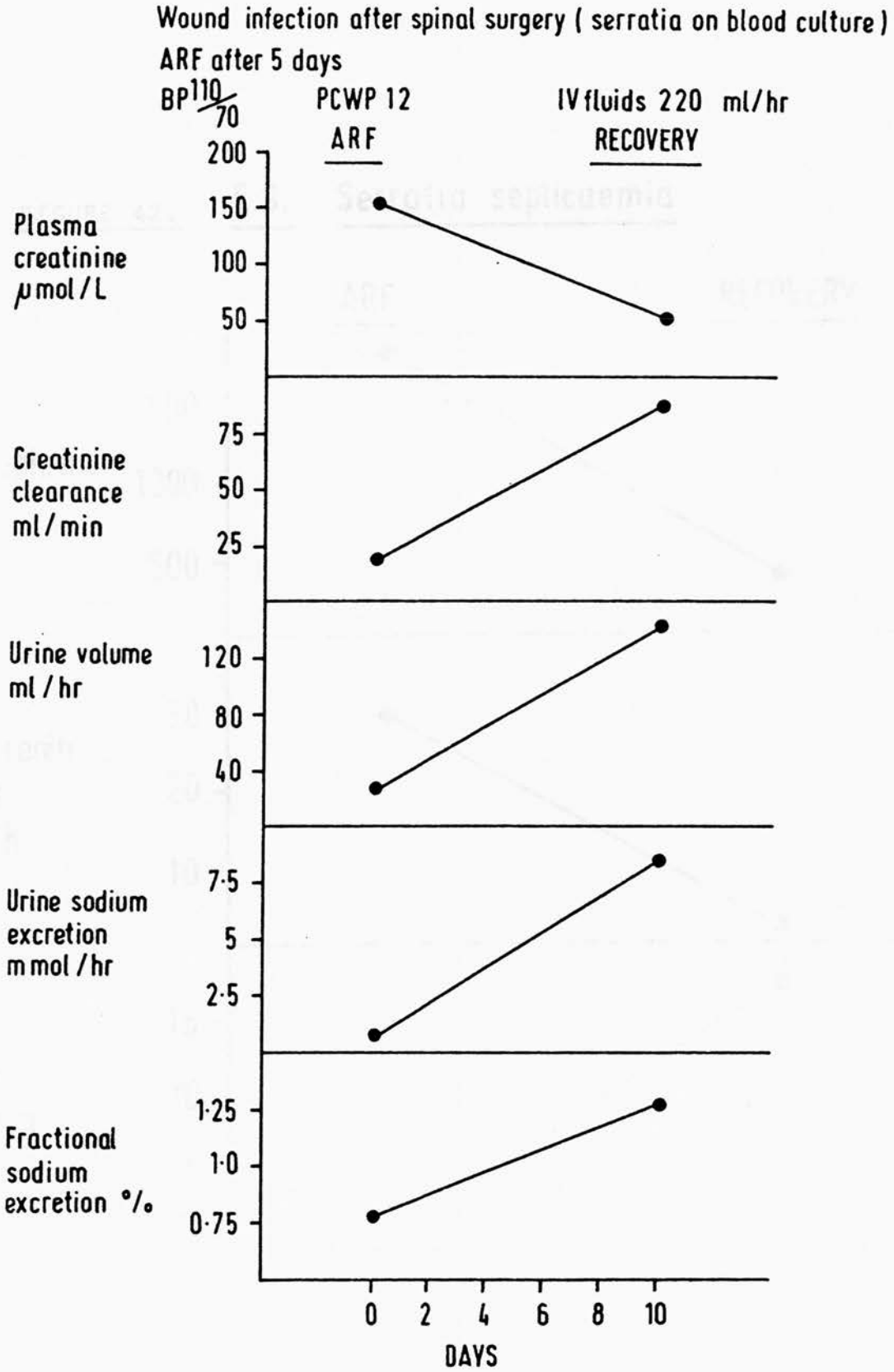
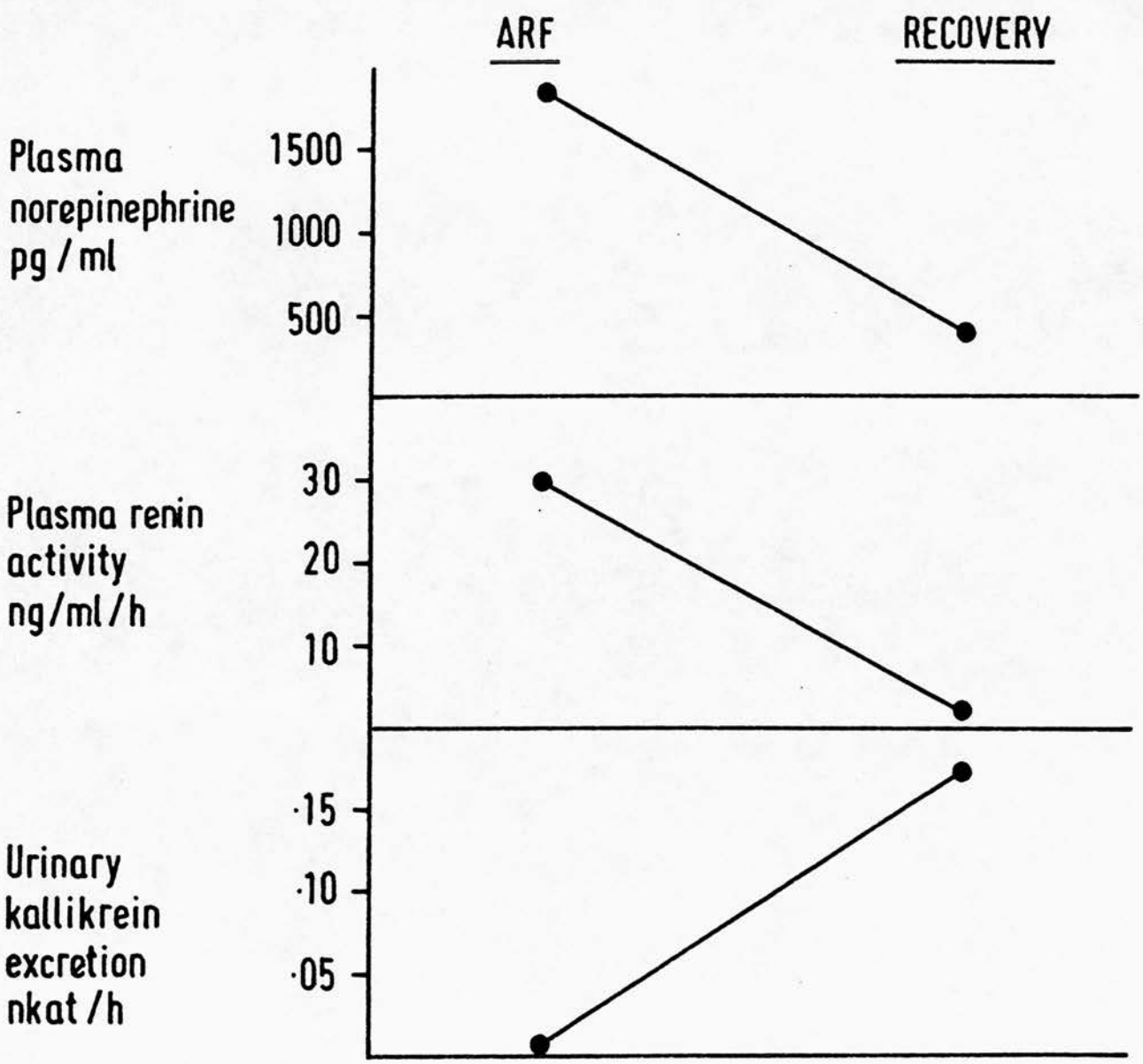


FIGURE 42. E.S. Serratia septicaemia



CHAPTER 10 INFLUENCE OF THE PLASMA PROTEIN CONCENTRATION ON URINARY KALLIKREIN EXCRETION

10.1. Introduction

In nephrotic syndrome (Chapter 7), cirrhosis (Chapter 8), and septic shock (Chapter 9), abnormal excretion of urinary kallikrein was observed in association with a reduction in plasma protein concentration, although the relationship was not qualitatively consistent. Experimental evidence suggests that the plasma protein concentration is an important determinant of glomerular and tubular function (Green, Windhager and Geibisch, 1974). For example, after acute volume expansion, the plasma protein concentration falls acutely; this is thought to increase renal plasma flow by reducing plasma viscosity, to increase GFR because of a lower oncotic pressure in glomerular capillaries, and to impair tubular sodium reabsorption because of a lowered oncotic pressure in proximal peritubular capillaries (Earley and Freidler, 1965; Grausz, Leiberman and Earley, 1972; Blantz, Rector and Seldin, 1974; Baylis et al, 1977; Baum and Berry, 1985). This last effect would increase distal delivery of sodium and water, and could thereby influence renin release from the macula densa and kallikrein release from the distal tubule (Hall and Guyton, 1976; Thurau, 1966). It was therefore of interest to examine the effect on renal function, and on kallikrein excretion, of an acute fall in plasma protein concentration. This can be achieved in animals by a form of plasmapheresis, involving removal of

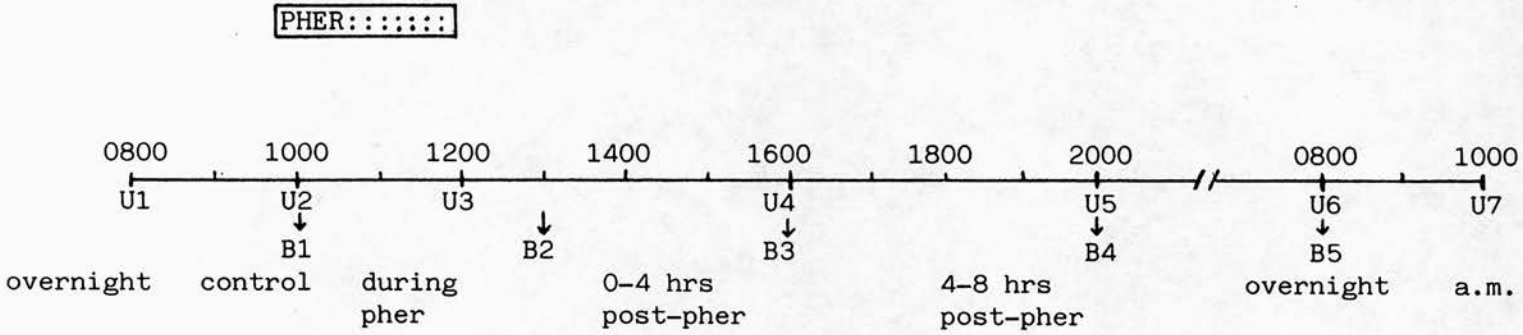
whole blood and reinfusion of packed cells and crystalloid (Kramer et al, 1982; Manning and Guyton, 1983). The fall in plasma oncotic pressure is associated with loss of fluid from the systemic circulation, which could influence renal function directly (Knox et al, 1983); to avoid this, central volume was monitored with a Swann-Ganz pulmonary artery catheter, and additional crystalloid infused to maintain the pulmonary artery occlusion pressure (PAOP).

10.2. Materials and Methods

7 healthy female sheep were studied, weighing 40-50 kg. On Day 1 they underwent brief general anaesthesia. An introducer was inserted in the external jugular vein for Swann-Ganz catheterisation and IV infusion; an arterial line was inserted in the carotid artery for blood removal and blood pressure monitoring; a urethral catheter was inserted. 400 ml blood was removed via arterial line, centrifuged, and the plasma separated. The cells were reconstituted with an equivalent volume of 0.9% sodium chloride, and the blood stored at -4 C. A background infusion of Ringers solution 100 ml/hr and 5% dextrose 50 ml/hr was begun and continued throughout the study. A 16 hour urine collection was performed.

The protocol on Day 2 is shown in Fig 43. The plasmapheresis procedure consisted of the removal of 3 units of 400 ml whole blood, with the simultaneous reinfusion of 3 units of cells reconstituted with saline, using the unit from Day 1 as the first reinfusion. Pulmonary artery occlusion

DAY 2



PHER - plasmapheresis - simultaneous removal of

- 3 x 400 ml whole blood, reinfusion of cells plus 0.9% saline (blood from Day 1 as first infusion)

- P.C.W.P., systemic BP and cardiac output monitored per 15 mins. R lactate infused to maintain P.C.W.P. at control values.

Figure 43. Details of protocol for Day 2 of plasmapheresis study.

pressure, systemic blood pressure and cardiac output were monitored at 15 minute intervals, and Ringers solution infused as required to maintain PAOP.

Blood samples were analysed for creatinine, total protein, albumin, sodium, potassium, osmolarity, haematocrit and PRA. Urines were analysed for creatinine, sodium, potassium, osmolarity and kallikrein.

Control studies were performed in 5 sheep, using the identical protocol, except that after centrifugation of the removed blood, the cells and plasma were reconstituted and reinfused together.

10.3. Results

In the saline replacement animals, the mean volume of plasma removed was 820 ml, and the mean volume of Ringers solution infused to maintain PAOP was 1300 ml/study. In controls, the mean volume of plasma separated and reinfused was 810 ml, and the additional Ringers solution infused during line flushing and priming was 250 ml. As shown in Fig 44, plasma total protein and albumin fell by 21% and 24% respectively during plasmapheresis. PAOP and mean arterial pressure were maintained during the study (Fig 45). There was no significant change in urine volume, urinary sodium excretion, FENa, or creatinine clearance (Figs 46,47,48). There was a significant rise in urine osmolarity, and a fall in free water clearance and plasma osmolarity (Figs 48,49), following plasmapheresis. PRA and urinary kallikrein excretion remained constant through the study (Fig 50).

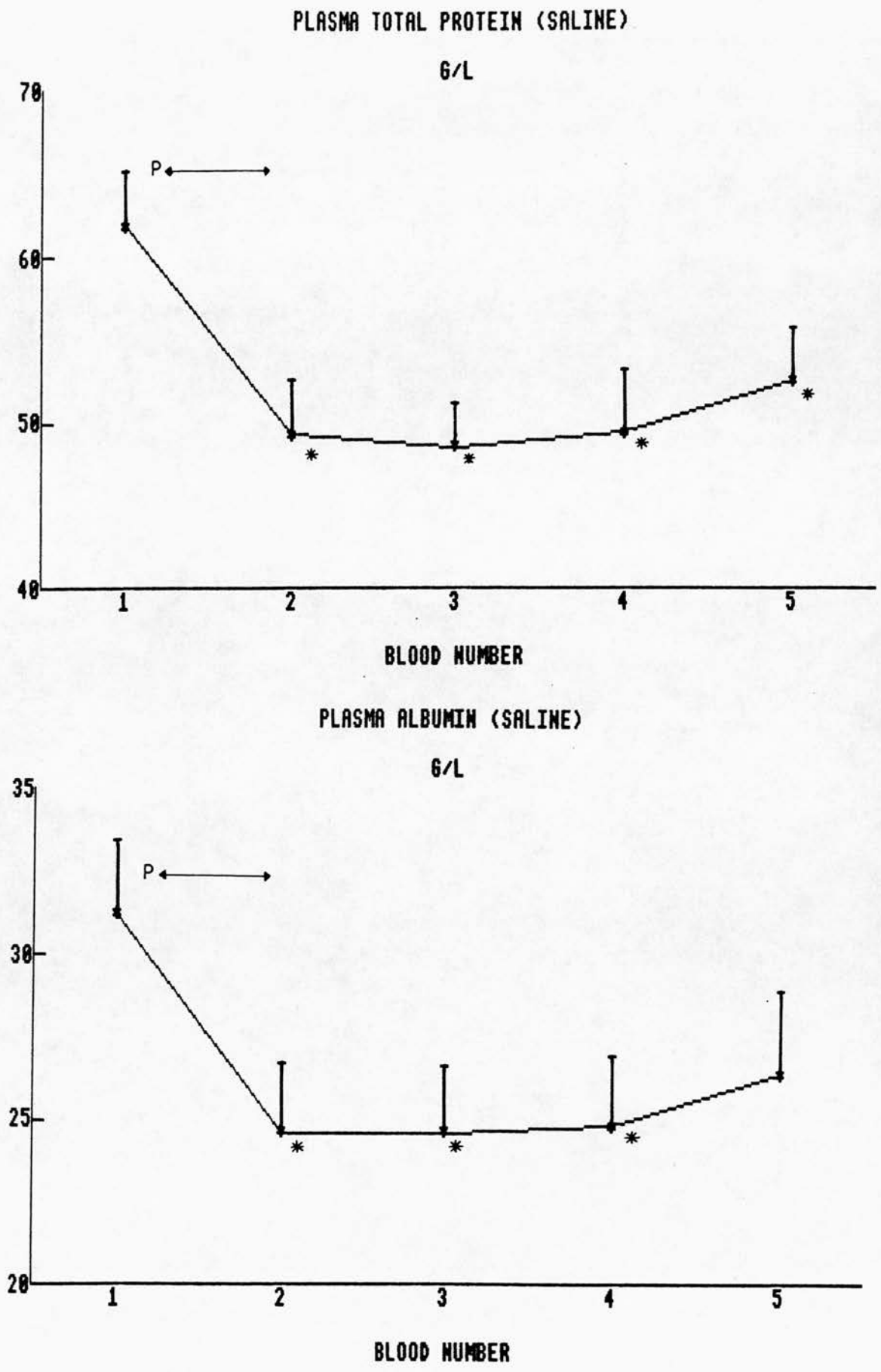


Figure 44. Plasma total protein and albumin; plasmapheresis study.

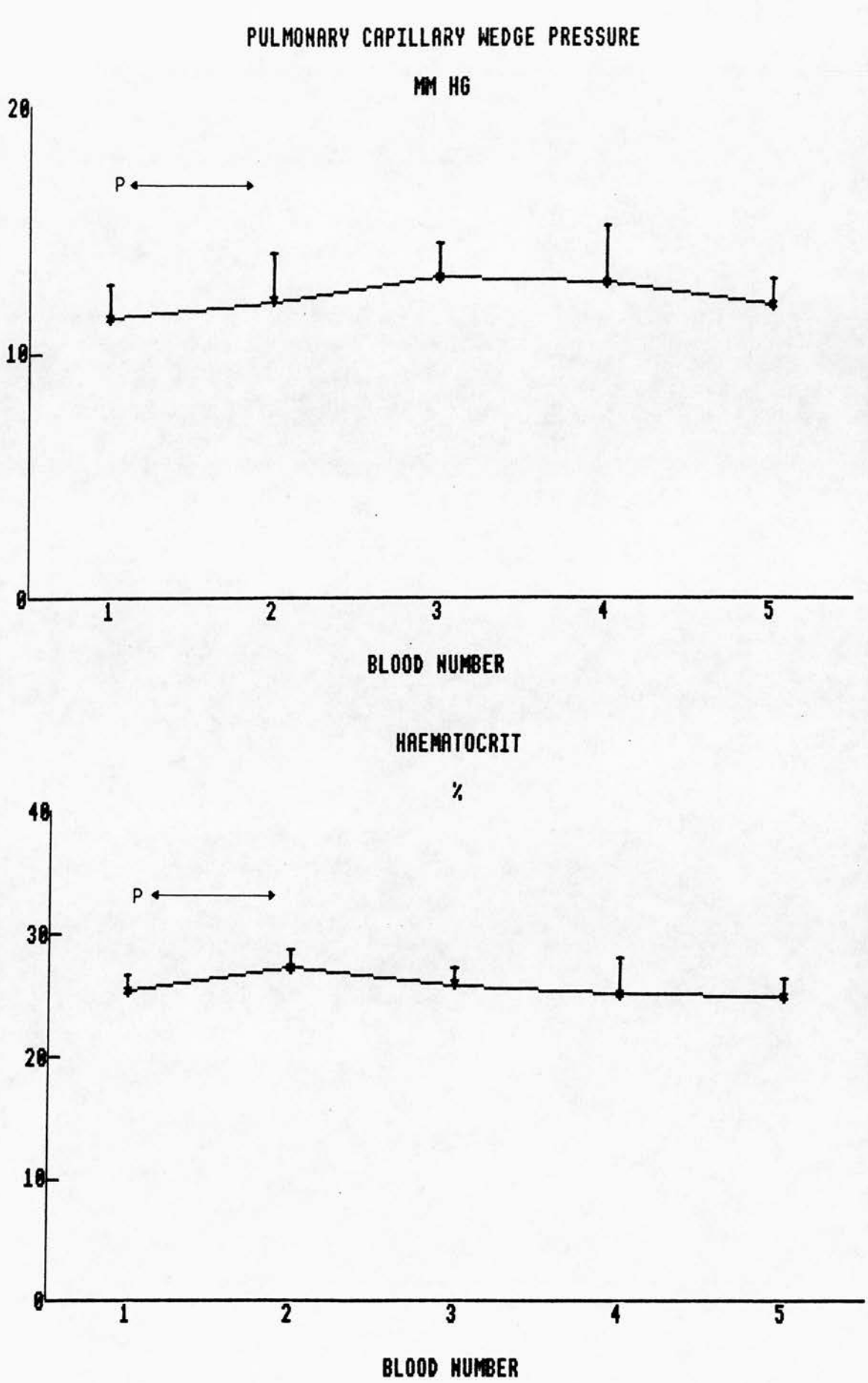


Figure 45. Pulmonary capillary wedge pressure and Haematocrit; plasmapheresis study.

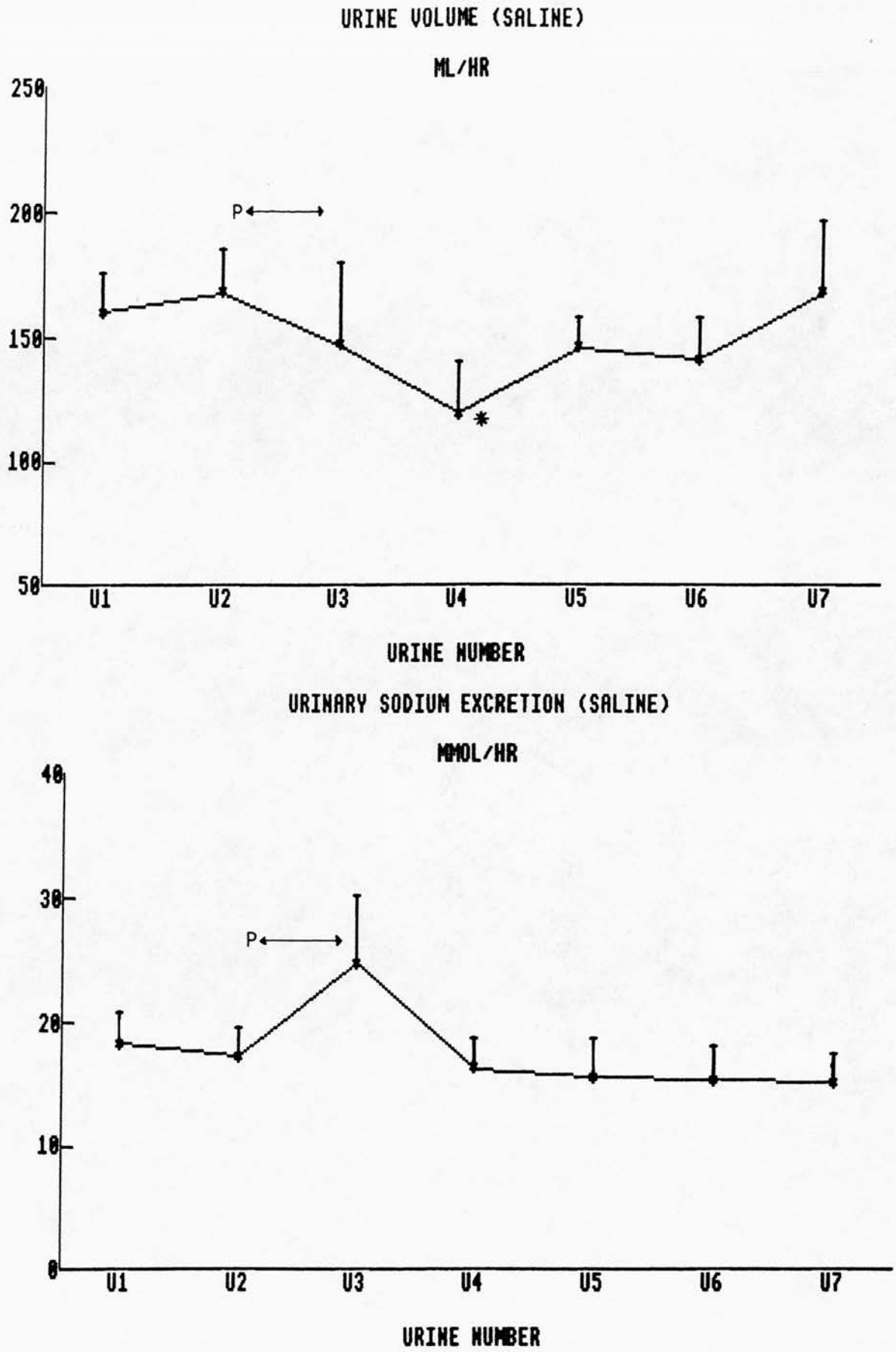


Figure 46. Urine volume and urinary sodium excretion; plasmapheresis study.

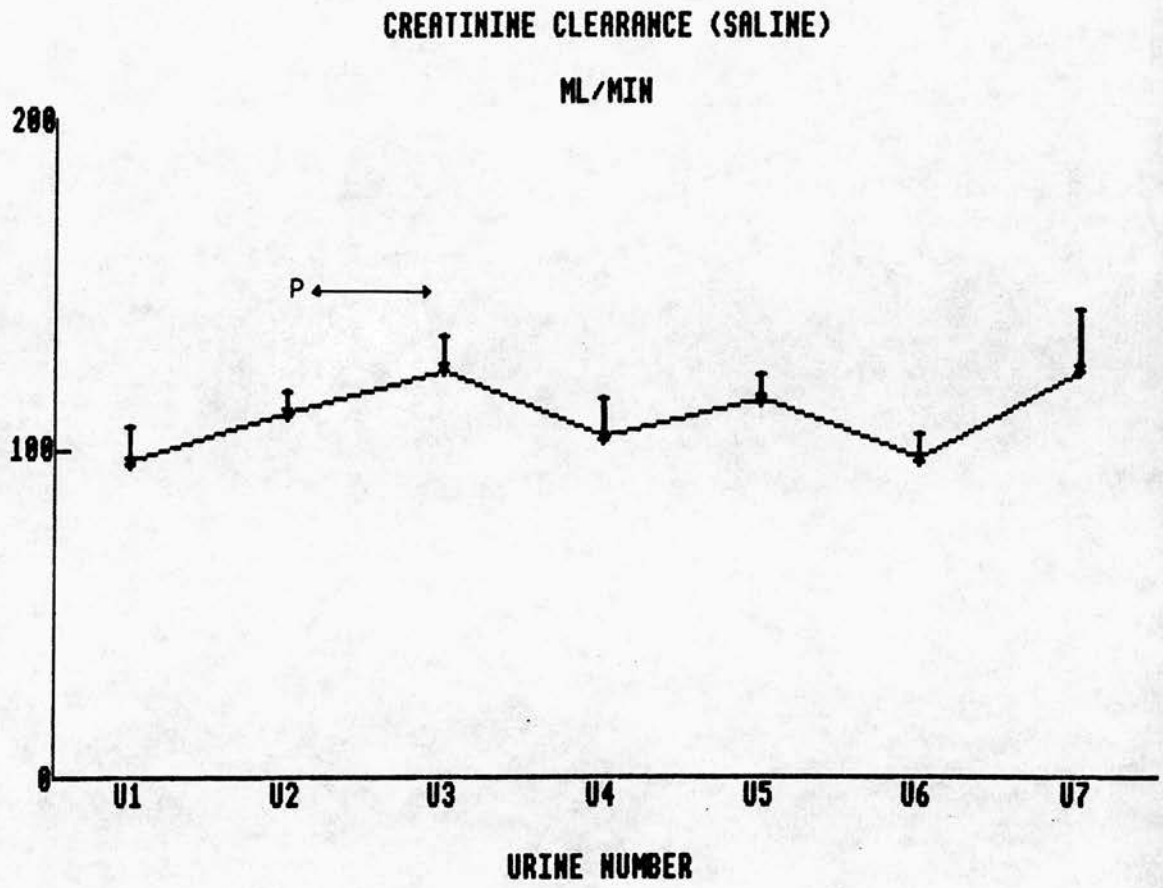


Figure 47. Creatinine clearance; plasmapheresis study.

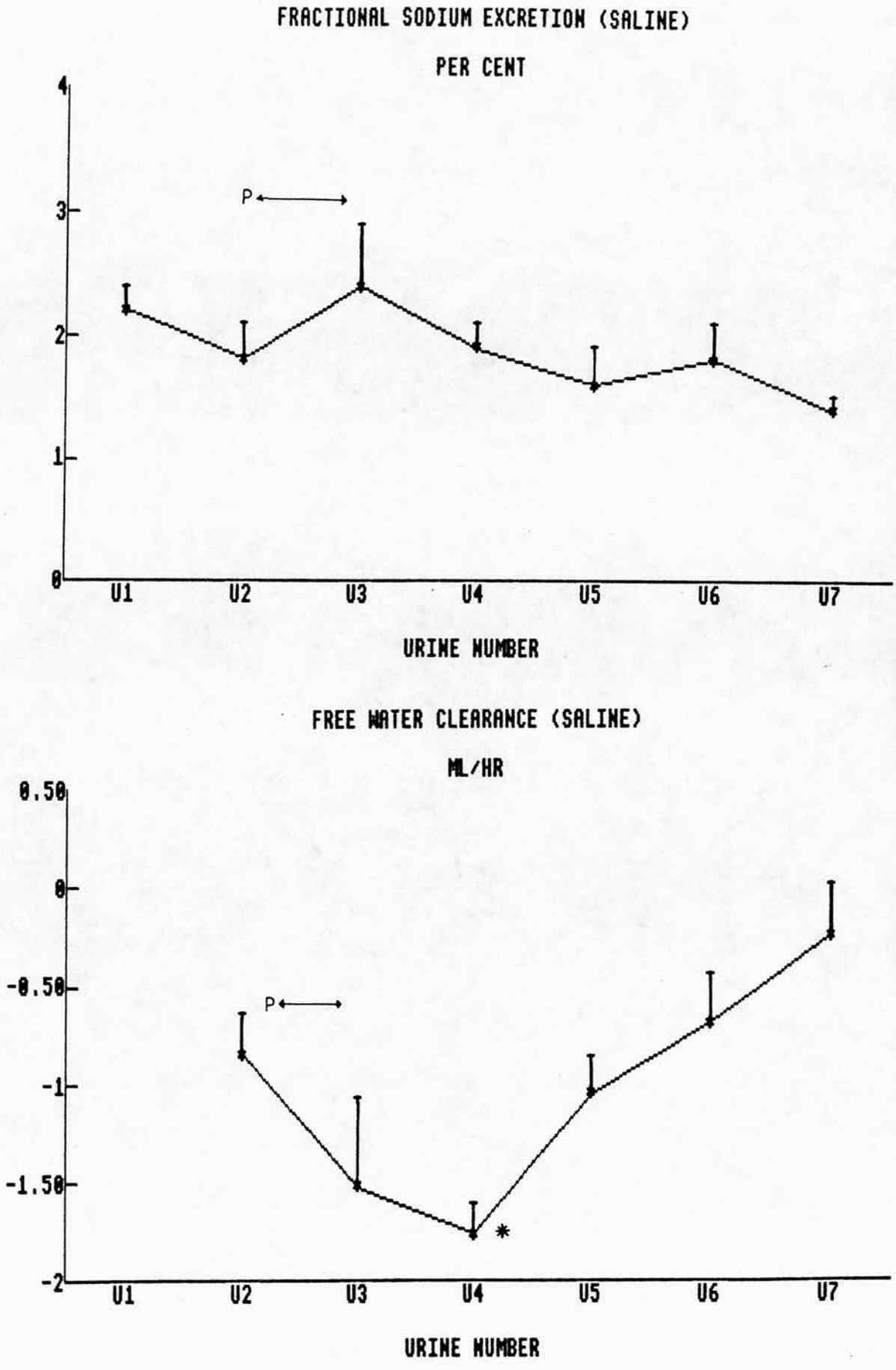
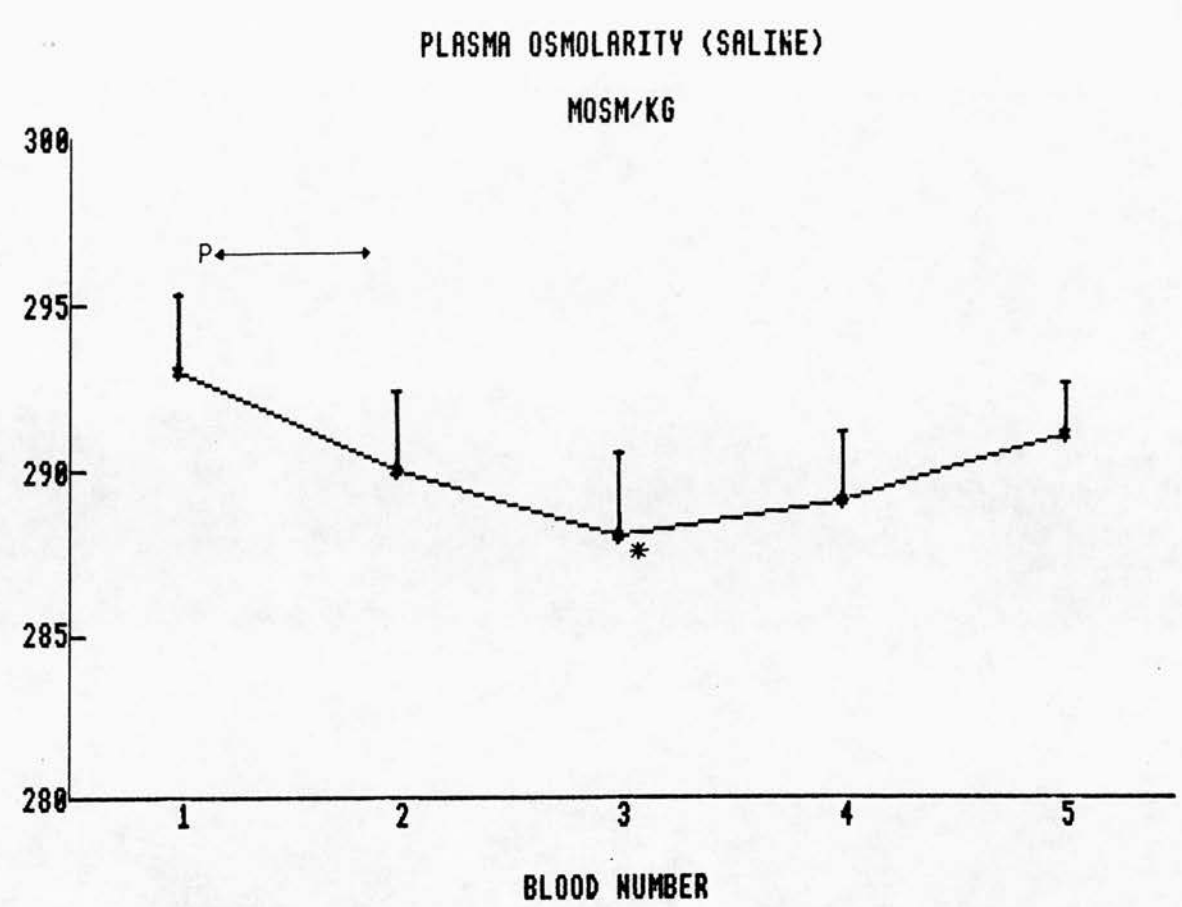


Figure 48. Fractional sodium excretion and Free water clearance; plasmapheresis study.



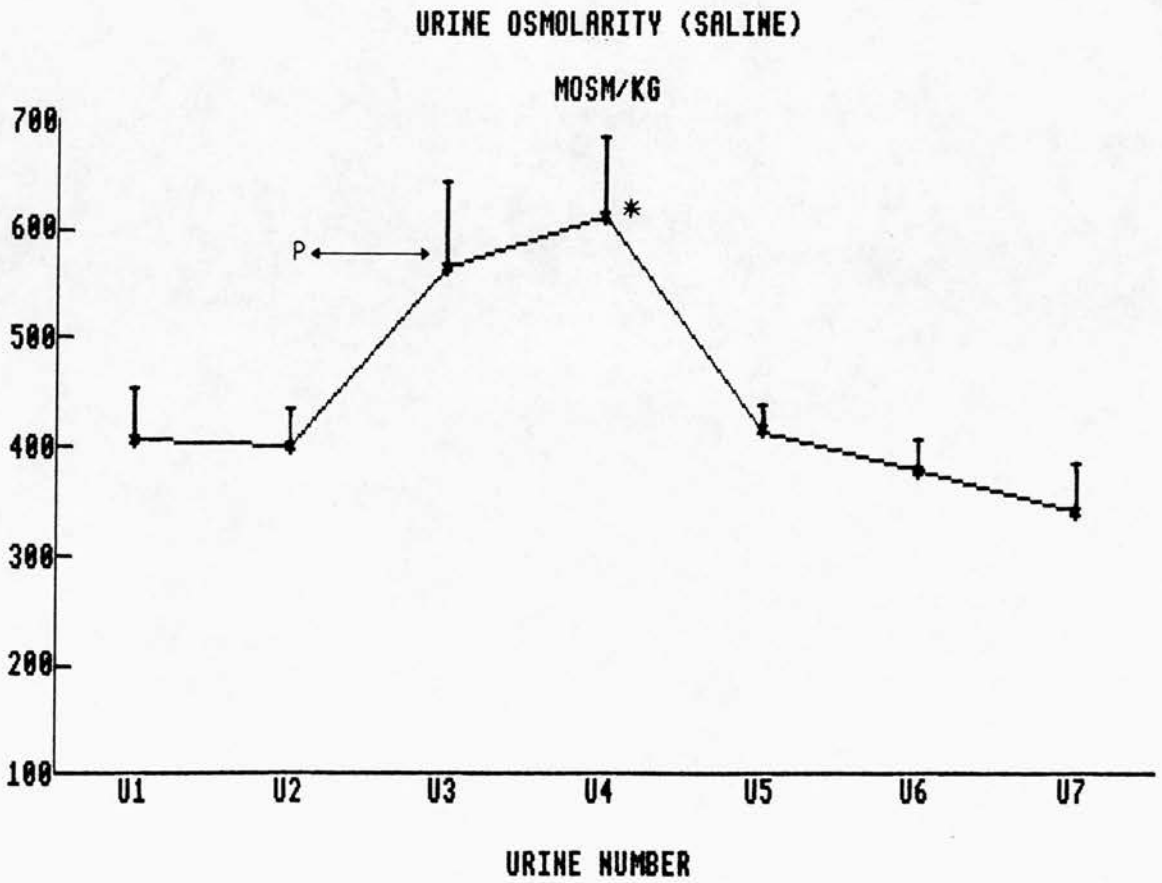


Figure 49. Plasma and urine osmolarities; plasmapheresis study.

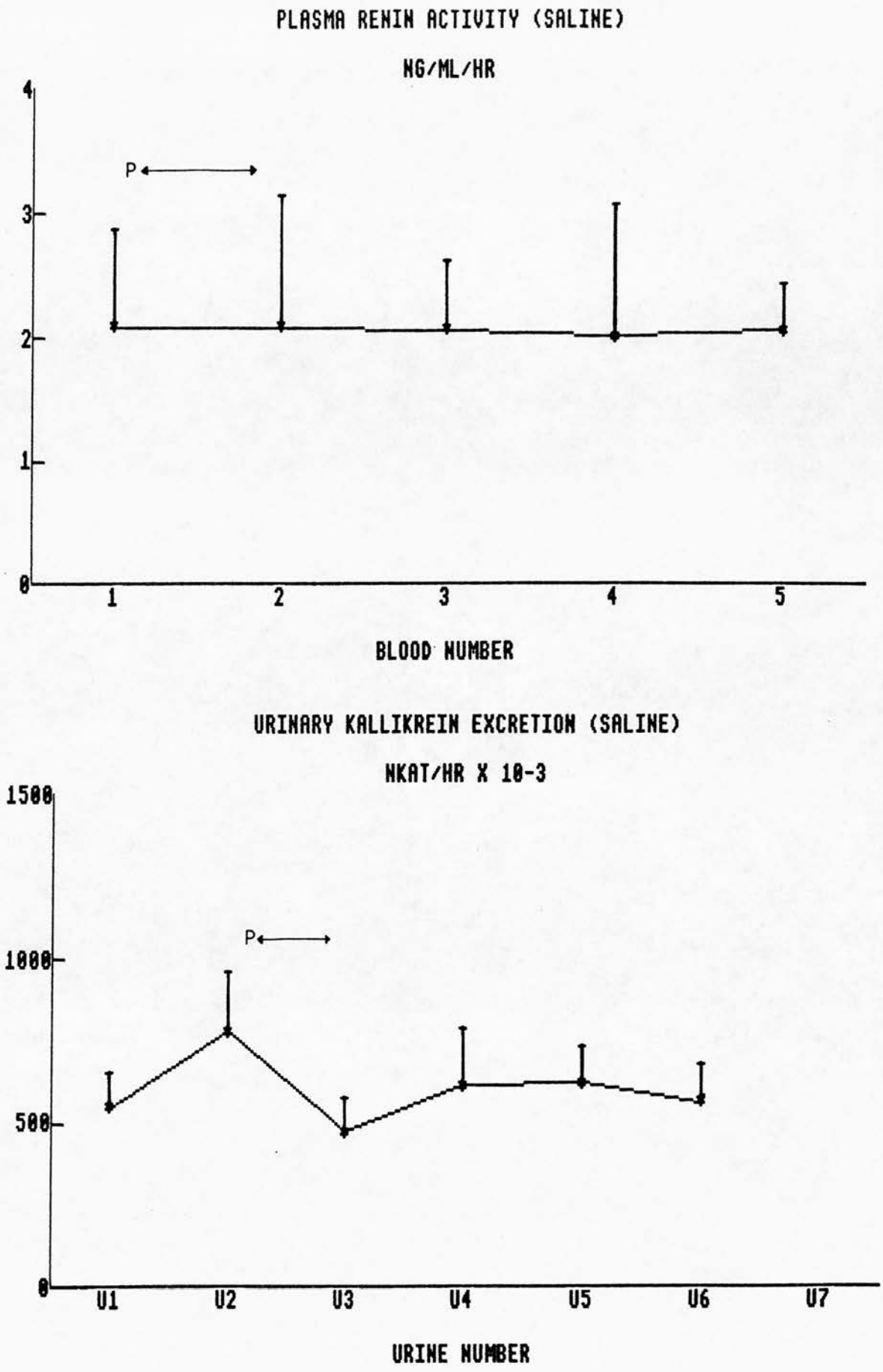


Figure 50. Plasma Renin activity and urinary kallikrein excretion; plasmapheresis study.

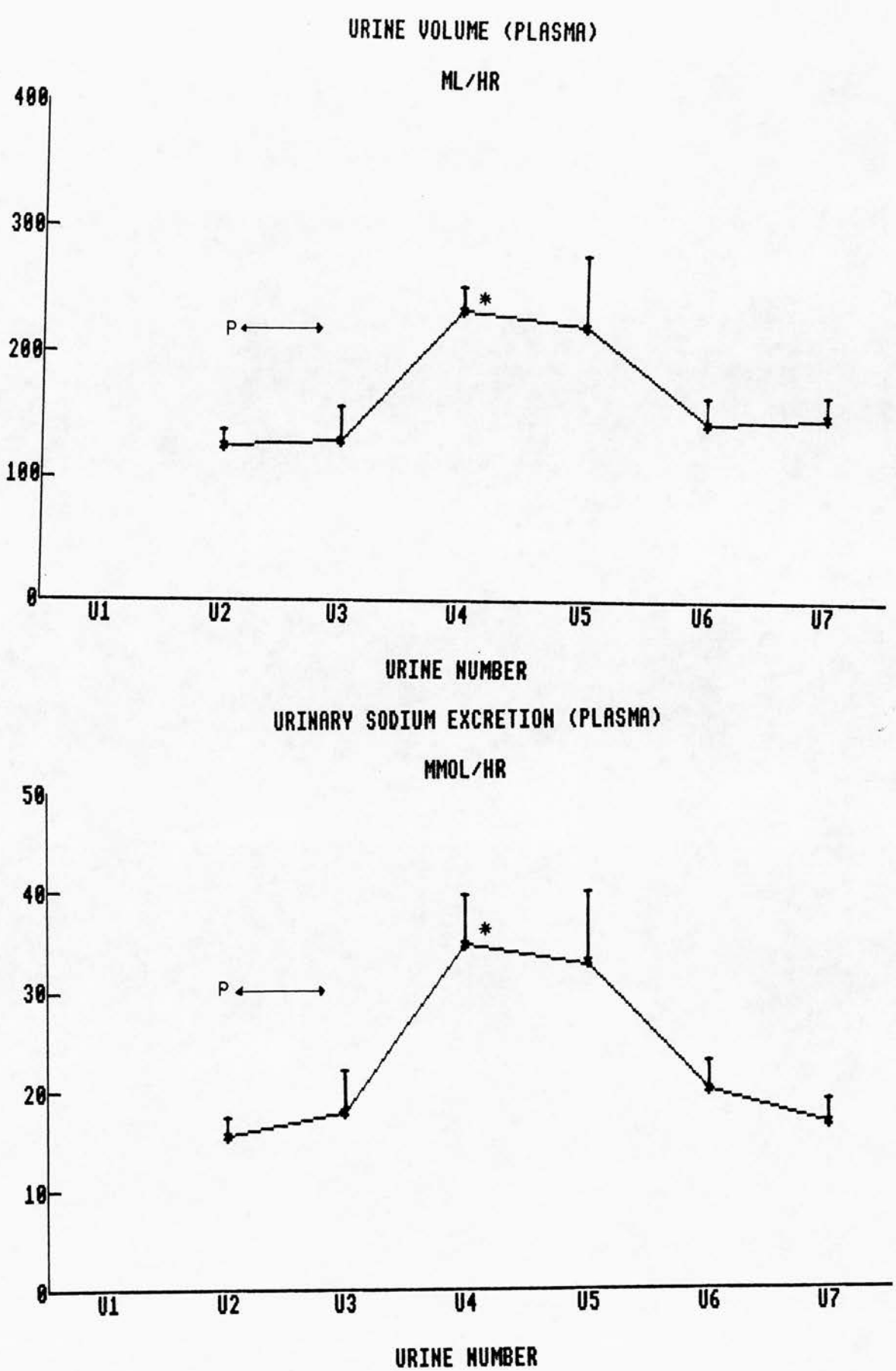


Figure 51. Urine volume and urinary sodium excretion in control studies (blood reconstituted and re-infused); plasmapheresis study.

In the control studies, there was a transient increase in urine volume and urinary sodium excretion after plasmapheresis, with no change in any other parameters (Fig 51).

10.4. Discussion

The modest natriuresis and diuresis seen in control experiments probably reflected the crystalloid administration which was required for line priming and flushing during the "sham" plasmapheresis procedure. Perhaps surprisingly, the acute reduction in plasma protein/albumin concentration in the plasmapheresis experiments was associated with no alteration in renal function, other than some changes in water handling. While we were not able to measure plasma AVP in sheep, the increase in urine osmolarity and fall in free water clearance, in face of a reduction in plasma osmolarity, is pathognomonic of AVP release (Skowsky and Fichman, 1984). This link between hypoproteinaemia and non-osmotic release of AVP is of considerable interest, and could relate to abnormalities of renal function in nephrotic syndrome and cirrhosis, as discussed in Chapter 8 (Wilkinson et al, 1979).

The absence of an effect on sodium excretion could have several explanations. The changes in proximal tubular function seen in micropuncture experiments may not occur in intact kidneys, or be confined to those superficial nephrons accessible to micropuncture (Baylis et al, 1977). Alternatively, there may be a compensatory fall in GFR,

although our creatinine clearance measurements argue against this, or the distal tubule may compensate by an increase in tubular sodium reabsorption (Ichikawa et al, 1983). The absence of any effect on renin release or urinary kallikrein excretion perhaps suggests that there is no major change in distal fluid delivery. The results suggest that the plasma protein concentration is not a major determinant of urinary kallikrein excretion, in the absence of changes in circulating volume. Alternative explanations for the altered kallikrein excretion in clinical hypoproteinaemic states, such as the nephrotic syndrome, are required.

CHAPTER 11 DISCUSSION

11.1. Plasma kallikrein-kinin system

Despite extensive study, the role of the plasma kallikrein-kinin system in health and disease remains unclear. Conceptually, it seems unlikely that such an effectively inhibited system has a major role in homeostasis. The plasma concentrations of kallikrein inhibitors such as C1 esterase inhibitor and ∝1-proteinase inhibitor greatly exceed that required for maximal inhibition under normal conditions (Ratnoff et al, 1969), and plasma kinins are almost completely destroyed during a single pass through the lungs (Regoli and Barabe, 1980). Rabito showed elegantly that glandular kallikrein reaching the circulation only influenced systemic haemodynamics if kininase activity was simultaneously blocked by captopril (Rabito et al, 1983). Under physiological conditions, kallikrein inhibition by aprotonin has no effect on systemic haemodynamics, or on renal function (Royston et al, 1987; Kramer et al, 1984). Similarly, the newly synthesised kinin receptor antagonists do not appear to influence blood pressure in normotensive or hypertensive animals (Waeber et al, 1988). While further work with these compounds is necessary for confirmation, it seems reasonable to conclude that the plasma kallikrein-kinin system is an "off" system under basal conditions, and does not have a significant role in physiology.

In contrast, it seems likely that the plasma kallikrein-kinin system is involved in the pathogenesis of at least

some disease states. Kinins are the most potent known vasodilator compounds, and are important mediators of local inflammatory responses and tissue injury (Marceau et al, 1983). Kallikreins are chemotactic for human neutrophils (Kaplan, Kay and Austen, 1971), and as described above, the plasma kallikrein-kinin system interacts importantly with the coagulation, fibrinolytic and complement systems, and with the arachidonic acid metabolic pathways (Kaplan, Meier and Mandle, 1977). Abnormalities of the plasma kallikrein-kinin system have been described in a wide variety of clinical states, but in most cases this consisted of a reduction in plasma prekallikrein. Current methodology makes it difficult to ascribe this to either increased activation and metabolism, or decreased synthesis, with any degree of certainty. We too have observed low plasma prekallikrein in disease states, namely septic shock and hepatic cirrhosis. We found spontaneous "kallikrein-like activity" against S2302, within the detection limit of the assay, to be extremely rare, such that the results are not amenable to detailed analysis, and have not been presented here. This does not exclude kallikrein-kinin system activation, since free kallikrein would bind to inhibitors in plasma, and has a plasma half-life of aproximately 5 minutes (Friberger et al, 1979). The reduced plasma prekallikrein must however be regarded as suggestive, rather than confirmatory, of plasma kallikrein-kinin system activation in these conditions.

The next step in defining the sigificance of these observations would be studies using inhibitors of the plasma

kallikrein-kinin system. It has recently been found that the dose schedule for aprotonin used in all previous experimental and clinical studies is too low to inhibit plasma, as opposed to glandular, kallikrein, by a factor of approximately 20 (Fritz and Wunderer, 1983; Dr F Schumann, Bayer FRG, personal communication). In any case, previous studies of aprotonin in cirrhosis are lacking, and studies in sepsis are of doubtful quality (Sumida, 1979; Rosengarten, 1979); in particular, none involved any measurements to ensure effective kallikrein inhibition. I am currently studying the effect of high-dose aprotonin in the ovine septic shock model described in Chapter 9; preliminary results suggest a marked beneficial effect on systemic haemodynamics, and in particular, prevention of progressive vasodilation.

The most potent trigger to the plasma kallikrein-kinin system is activation of Hageman factor by endotoxin. Endotoxin in plasma is increased in both septic shock and cirrhosis (Wardle, 1982; Papper, 1982). Interestingly, these two conditions also have in common a tendency to systemic vasodilation, and a pattern of renal function involving sodium retention despite normal renal plasma flow. This pattern of renal function has been ascribed to a low "effective plasma volume" (Nicholls et al, 1986). It is possible that kallikrein-kinin activation promotes a fall in systemic vascular resistance; the subsequent fall in blood pressure is minimised by reflex activation of pressor

systems such as the sympathetic nervous system and renin-angiotensin. This induces similar changes in renal function to "genuine" plasma volume contraction. Plasma volume expansion would tend to promote further vasodilation, partly via release of the vasodilator prostaglandin PGI2 from endothelium (Hamilton, Rosza and Hutton, 1981); kinins could exaggerate this effect by activation of Phospholipase A2 leading to increased arachidonic acid substrate (Regoli and Barabe, 1980). This would explain the "volume-resistance" seen in both clinical contexts. A study of patients after instrumentation of the urinary tract found associations between endotoxaemia, a reduction in plasma prekallikrein concentration, and a fall in systemic vascular resistance (Robinson et al, 1975). While this hypothesis (Figure 40) is speculative, it is in keeping with known physiological and pathophysiological mechanisms; it is also testable with the availability of kallikrein and, more recently, kinin inhibitors. It is also relevant to the introduction of anti-endotoxin antibodies and purified α1-proteinase inhibitor for prophylactic and interventive use.

11.2. Renal kallikrein-kinin system

11.2.1. Anatomical location and functional implications

The findings described in Chapter 5, that kallikrein is located in the glomerular peripolar cell in addition to the cells of the distal tubule, are of considerable importance. Confinement of renal kallikrein to cells of the distal nephron would not necessarily preclude an effect on

glomerular or proximal tubular function, or on renal vascular tone, since release of immunoreactive kallikrein into the renal interstitium and renal lymph has been demonstrated (Proud et al, 1983). Nevertheless, such release, with subsequent uptake by lymphatics, would be a relatively inefficient and variable mechanism in biological terms, and would not facilitate precise interaction with other intrarenal regulatory systems.

In vitro, kinins and prostaglandins antagonise the actions of AVP on distal tubular and collecting duct epithelium, and some in vivo studies have found correlations between kallikrein and water excretion (Schuster, Kokko and Jacobsen, 1984; Mills and Ward, 1975). It is tempting to speculate that this is the primary function of the distally located kallikrein and the kinin which is generated at this locus. Kramer and Dusing have shown that aprotinin markedly potentiates the effects of DDAVP on urine flow and urine osmolarity in the rat (Kramer at al, 1984). It would be predicted in this case that the effect of distally released kallikrein on water handling would depend critically on the prevailing AVP concentration. This has not been tested experimentally, and detailed studies of the relationship between AVP and kallikrein effects on collecting duct function would be of interest.

In all the studies described in this thesis, the correlation of kallikrein excretion with sodium excretion has been stronger than that with water excretion; this aspect of the findings is discussed below. Sodium excretion is dependent

on glomerular filtration rate, proximal and distal tubular sodium reabsorption, and the function of the tubulo-glomerular feedback mechanism (De Wardener et al, 1978; Licht and Danovitch, 1983). The juxtaglomerular apparatus is centrally involved in the regulation of each of these parameters (Fray, 1980). The finding of kallikrein at this site therefore greatly increases the potential role of kallikrein in the regulation of sodium excretion. The stimuli to release of kallikrein from cells are imprecisely defined (see introduction). The juxtaglomerular apparatus is richly innervated by sympathetic nerve fibres, incorporates a baroreceptor-like mechanism, and also responds to changes in volume and composition of distal tubular fluid (Dzau, Burt and Pratt, 1988). It is of interest that in the isolated perfused kidney, there is a direct association between the renal perfusion pressure and kallikrein excretion (Bevan, McFarlane and Mills, 1974), and that both renal nerve stimulation and infusion of noradrenaline appear to supress kallikrein excretion (Albertini et al, 1981; mills, 1980). The potential importance of renin-kallikrein interactions at the juxtaglomerular site is discussed below. Injected radio-labelled kallikrein is largely degraded before reaching the final urine (Mills, Paterson and Ward, 1975; Rabito et al, 1985); some of this metabolism is ascribed to uptake by the proximal tubule. It must therefore be asked to what extent measurement of urinary kallikrein gives information regarding release from peripolar cells.

The pattern of immunoperoxidase staining shown in Chapter 5 suggests that quantitatively, most of the kallikrein in kidney is in distal tubular cells, and it therefore seems likely that most of the kallikrein in urine derives from this source. Micropuncture studies have suggested that most kininogenase activity enters the tubular lumen at the level of the distal tubule and collecting duct (Scicli et al, 1976). It is possible, however, that the peripolar cells and the kallikrein-containing distal tubular cells react in a similar fashion to relevant stimuli, in which case the urinary kallikrein excretion rate would be a measure of release at both sites. It even conceivable that proximal kallikrein release might in itself tend to stimulate release from distal sites, by increasing delivery of fluid to the distal tubule. Many of the situations in which urinary kallikrein excretion is increased are characterised by increased distal fluid delivery; these include acute volume expansion, infusion of renal vasodilators, and administration of loop diuretics, as discussed previously. Further studies are required to confirm or refute this hypothesis.

11.2.2. Relationship to sodium excretion

A fundamental question regarding the renal kallikrein-kinin system concerns its influence on sodium excretion. Since the pioneering work of Mills and colleagues in the 1970's, it was generally held that kallikreins and kinins facilitated sodium excretion (Mills, 1982). In 1983, Marks and Keiser challenged this assumption, and suggested that kallikrein-

kinin was a sodium retaining system (Marks and Kieser, 1983). Their main evidence was a study involving modest volume expansion with normal saline in rats. As in our human studies, they observed an early increase in urinary kallikrein excretion, peaking within 1 hour. Thereafter, however, UKallV fell, and at times was lower than control values. There was no correlation between UNaV and UKallV. Adrenalectomy reduced basal UKallV by 50%, but the response to volume expansion was unaffected. They also took account of previous work, showing that kallikrein stimulates sodium transport in the isolated toad hemi-bladder (Orce, Castillo and Margolius, 1981).

Against this rather tenuous evidence must be set a considerable body of data favouring a natriuretic effect of renal kallikrein-kinin activation. While none of these experiments is conclusive in itself, I would suggest that overall they suffice to make any other view untenable. As described in Chapter 1, infusion of kinins into the renal artery increases sodium excretion (Mills, 1979); cross-clamping experiments suggest that this is primarily a renal vasodilator effect, although the action of intra-luminal kinins may be different (Kauker, 1980). Systemic infusion of glandular kallikrein produces a shock-like syndrome, and the effects on renal function are difficult to interpret (Mills, 1979). Both the kallikrein inhibitor aprotonin and anti-kinin antibodies reduce sodium excretion, although the aprotonin effect is only seen in volume expanded animals

(Kramer et al, 1984). Kallikrein excretion increases in response to increased dietary sodium, and to acute infusion of isotonic sodium chloride. Lewis et al (1988) recently found no increase in kallikrein excretion in man after a 2 litre saline infusion, whereas the study described in Chapter 5 clearly shows such an increase after a 3 litre infusion, suggesting that there may be a threshold for the acute effect of volume expansion on UKallV. This would also explain some other divergent results in animal studies of this response. It should be noted that in the studies described in this thesis, the correlation between urinary kallikrein excretion rate and sodium excretion rate was always positive rather than inverse. This was true in situations ranging from normal man under basal and stimulated conditions, through to man and experimental animals in the presence of renal and extrarenal disease, and contrasted to the correlations between PRA and UNaV, which were always inverse. The statistical significance of the UKallV / UNaV correlation was usually weak; this might be expected in view of the multitude of factors influencing sodium excretion and the imprecise manner in which urinary kallikrein excretion probably reflects intrarenal events (Fuller and Funder, 1987). Accepting that studies of correlation must be interpreted with caution, it would be expected that if the renal kallikrein-kinin system was either antinatriuretic or neutral in relation to sodium excretion, an inverse or absent correlation would have occured in at least some of these experiments. Overall, it

seems clear that the renal kallikrein-kinin system is indeed a natriuretic mechanism, although the precise relationship with other natriuretic and sodium-retaining systems must be defined.

11. 2. 3. Relationship to the renin-angiotensin system

The concept that the renal kallikrein-kinin system and the renin-angiotensin system are functionally linked is not new. However, in these studies, use of modern immunocytochemical methods and powerful computer-based statistical techniques has shed additional light on this relationship.

Writing in 1975, Pisano pointed out the strikingly close homology in the organisation of these two systems (Fig 16). He suggested on this basis that they had evolved in parallel as counter-regulatory mechanisms. In 1977, Levy et al first calculated the ratio of PRA to urinary kallikrein excretion, in a study of hypertensive patients and their relatives (Levy et al, 1977). They found a close correlation between this ratio and renal blood flow, which was reduced in the hypertensive subjects. They did not comment on any other correlations, such as with sodium excretion. Mills had previously suggested that one possible action of the renal kallikrein-kinin system might be to protect the renal vasculature against the vasoconstrictor action of locally generated Angiotensin II (Mills et al, 1972). It was suggested that renal release of kallikrein was under dual control. Sodium loading increased UKallV, as described above, possibly through a volume or pressure-sensing

mechanism. UKallV was also increased, however, during sodium restriction or hypotensive haemorrhage; this could be secondary to activation of the renin-angiotensin system. Studies infusing Angiotensin II into the renal artery tended to support this idea, with an increase in UKallV coinciding with development of resistance to the vasoconstrictor effect (McFarlane, Adetuyibi and Mills, 1974). A further boost to interest in this relationship came when Sealey and Laragh described the ability of renal kallikrein to activate prorenin (Sealey et al, 1978) ; aprotonin was shown to reduce captopril-stimulated renin release, and bradykinin was also shown to stimulate renin release from kidney slices (Beierwaltes, Prada and Carretero, 1985). Subsequently, however, doubt was cast on the significance of the studies of Sealey et al, and this area has received little attention in recent years (Hsueh, 1980).

Teleological considerations would favour a link between the kallikrein-kinin system and the renin-angiotensin system. In all our studies, the correlation of PRA with sodium excretion was stronger than that between UKallV and sodium excretion, suggesting that the renin-angiotensin system has "primacy" in this area. In evolutionary terms the first requirement of land-based organisms would be a mechanism to retain sodium and water and preserve ECF volume. Later, as access to salt and water increased, a counter-regulatory mechanism to prevent sodium overload, hypertension, and heart failure would be advantageous. Such secondary evolution would fit with the relative paucity of peripolar

cells in most species. It is of interest that sheep, which exist in a low-sodium environment, have a very prominent and cellular juxtaglomerular apparatus, readily increase PRA in response to stimuli, and have the largest number of peripolar cells in species so far examined.

There are obviously a number of other candidates for the type of counter-regulatory action described above; these include renal prostaglandins, dopamine, atrial natriuretic peptide, and endogenous oubain-like Na-K ATPase inhibitors. It is likely that some or all of these do function in concert with the renal kallikrein-kinin system, either as a "cascade" (Mills, 1982), or in a "network" fashion. The kallikrein-kinin system differs, however, in that a close anatomical relationship with the "control centre" of the renin-angiotensin system has now been demonstrated; such a localisation would be predicted to optimise rapid and effective functional linkage. The observed correlations between the PRA/UKallV ratio and sodium excretion in these studies, in some cases as high as r=0.91, suggest that the relative activity of the renin-angiotensin system and the renal kallikrein-kinin system is a powerful determinant of sodium excretion. This conclusion is subject to various caveats. Statistical association does not prove causality. However, when a correlation recurs with a high degree of significance in a large number of independent experimental situations, as here, it cannot be ignored. The use of derived ratios is often regarded with scepticism.

Nevertheless, this ratio is firmly based on anatomical, biochemical and physiological considerations, as reviewed above, rather than eclectic considerations. It might be suspected that the observed correlations were a result of combining two relatively weak correlations into a single strong one. In fact, the combination of two variables in this manner is equally likely to weaken or destroy any correlation, as to strengthen it (Dr Robert Elton, Dept of Medical Statistics, University of Edinburgh, personal communication). In every case described here, the degree of correlation of the ratio with sodium excretion was greater than PRA or UKallV alone. If a single agent was found to correlate with UNaV to this extent, there would be little doubt about its recognition as an important determinant of sodium excretion.

If, as suggested by the results described, up to 80% of the variability in sodium excretion can be accounted for by changes in the PRA/UKallV ratio, it is tempting to speculate that other factors which influence sodium excretion might operate by modulation of this central relationship, rather than functioning in a parallel fashion. For example, both intrarenal dopamine and atrial natriuretic peptide tend to supress renal renin release, but have no supressive effect on kallikrein release (Brownlee et al, 1986; Firth, Raine and Ledingham, 1989); they would therefore be predicted to lower the PRA/UKallV ratio, in association with natriuresis. These mediators of course have their own receptors in the kidney; however, the extent to which kallikrein-kinin

blockade inhibits their renal actions is not known. Conversely, renal nerve stimulation will increase PRA and tend to lower UKallV, in association with a reduction in urinary sodium excretion (Albertini et al, 1981). An outline of this hypothesis is shown in Fig 52.

At the time of writing, studies of the renin-kallikrein-sodium relationship have proved informative in the nephrotic syndrome, in hepatic cirrhosis, and in acute renal failure due to sepsis. Collection of similar data is in progress in essential hypertension (Levy et al, 1977), in diabetes mellitus (Mayfield et al, 1985), and in cardiac failure. In addition to aiding understanding of pathophysiology, these studies may have therapeutic implications. For example, in sodium-retaining acute renal failure, the ideal intervention would be an agent which supressed the renin-angiotensin system while potentiating or maintaining kallikrein-kinin activity. Captopril, which fulfills the former requirement, has been used in experimental studies of ARF, but has proved disappointing; for example, in the sheep sepsis model, it was associated with rapid deterioration in renal function (Dr FJ Walker, personal communication). It is therefore of interest that we observed profound reductions in UKallV during captopril therapy. Loop diuretics, which stimulate UKallV (Overlack et al, 1982), have also proved of little value in ARF (Werb and Linton, 1979); this could reflect the concomitant stimulation of the renin-angiotensin system which they are known to cause (Mackay et al, 1985).

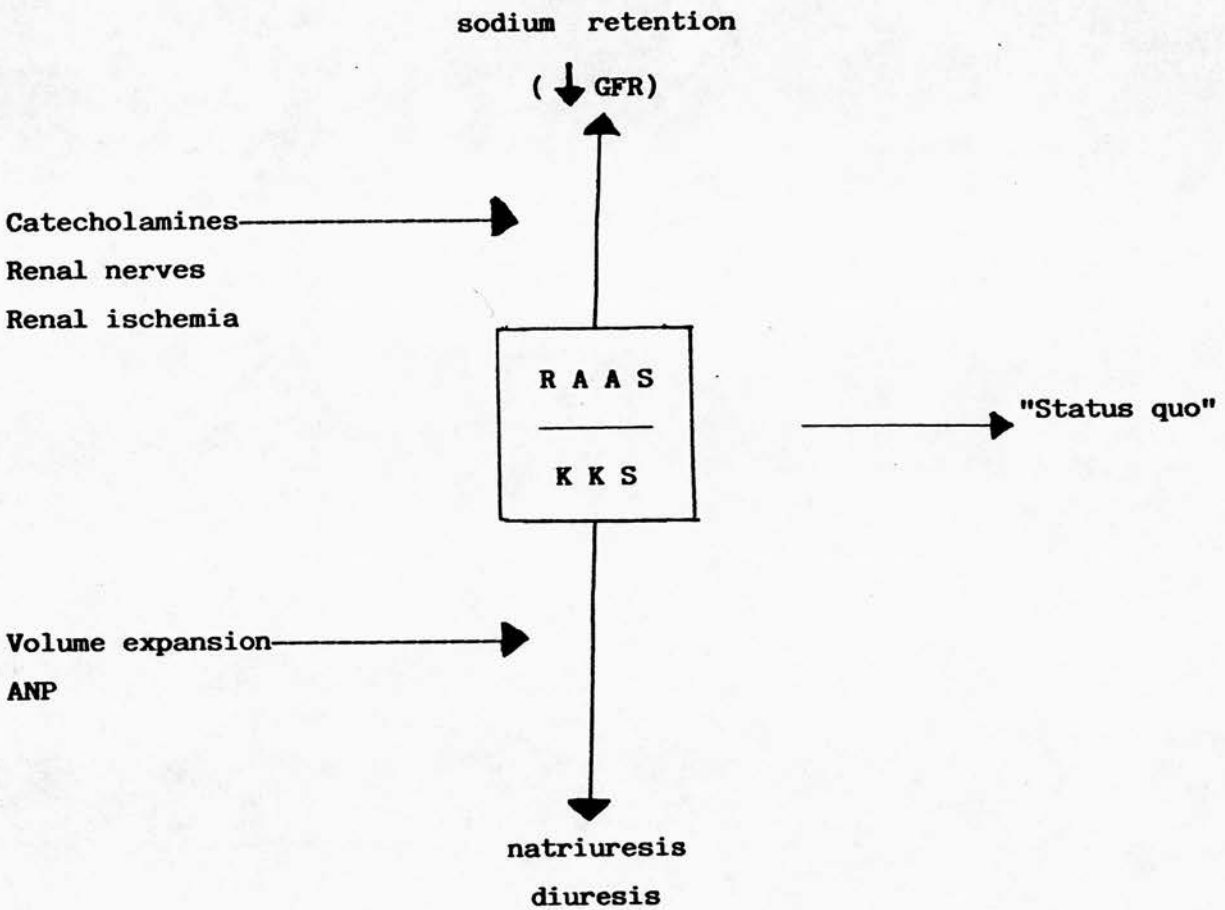


Figure 52. Diagram of hypothetical counter-regulatory linkage between renin-angiotensin and kallikrein-kinin systems in the kidney.

Dopamine, which does appear of value, would be expected to lower the PRA/UKallV ratio, as discussed above. ANF infusion is likely to have a similar effect, and early results in ARF are encouraging (JD Conger, personal communication).

11.2.4 Kallikreins and kinins as mediators of renal inflammation

In this and previous studies of the renal kallikrein-kinin system, attention has focussed on the role of these compounds in the regulation of renal function, rather than on a possible role as mediators of intra-renal inflammation. In this and a few previous studies, no abnormality of urinary kallikrein excretion was found in glomerulonephritis without heavy proteinuria (Glasser and Michael, 1976). This does not exclude an effect of kallikreins and kinins in the pathogenesis of renal disease in these patients; however, it seems likely that in most circumstances, the ability of the kidney to degrade kallikreins and kinins prevents such an effect. In contrast, urinary kallikrein excretion is elevated in most patients with nephrotic syndrome, sometimes to extremely high values. Taken in conjunction with the known potent effect of kinins on capillary permeability (Regoli and Barabe, 1980), and the known ability of intra-renal kallikrein infusion to cause heavy proteinuria (Murakami, Hori and Masamura, 1968), this finding engenders speculation that the renal kallikrein-kinin system is of pathogenetic importance in this syndrome. Recently, increased salivary kallikrein has been

observed in patients with chronic recurrent parotitis, a disease of previously unknown aetiology. In addition, patients with this condition were found to respond rapidly and completely to systemic infusion of high dose aprotonin (Maier et al, 1985). The parallel with nephrotic syndrome is readily apparent, and studies of the effect of aprotonin in nephrotic syndrome are in progress. Murakami in 1968 claimed to have abolished proteinuria in patients by giving aprotonin, but this claim has never been substantiated or repeated. The nature of the stimulus to kallikrein release in nephrotic syndrome, and the mechanism whereby the renal kallikrein-kinin system "escapes" from inhibition in this condition, are unclear, although important inhibitors such as α1-proteinase inhibitor, C1-esterase inhibitor and endogenous aprotonin will be lost in urine in nephrotic syndrome, perhaps leading to a "vicious circle" phenomenon. It is likely that these proteinase inhibitors will soon be available for therapeutic use as a result of recombinant DNA technology.

11. 2. 5. Future trends

The studies described in this thesis have left many questions unanswered. This has, however, been true of all previous studies in this field, and over the years many eminent researchers have abandoned study of the kallikrein-kinin system without reaching definitive conclusions. Recent developments have opened exciting new avenues of research. The availability, after years of searching for these

compounds, of specific kinin receptor antagonists, should lead to an increased understanding of kinin actions in the kidney and elsewhere (Vavreck and Stewart, 1987). Advances in methodology and assay technology, such as the use of monoclonal antibodies in specific enzyme-linked immunoadsorbent assays for kallikrein, offer improved assessment of enzyme levels in biological fluids. Perhaps most exciting is the cloning of kallikrein genes in mouse, human and other species, and the availability of complementary DNA probes for these genes (Mason et al, 1983; Bell et al, 1984). I am currently involved in studies correlating urinary kallikrein excretion with the presence of restriction fragment length polymorphisms around the kallikrein genes in patients with contrasting risks of hypertension. I am also developing techniques of in situ DNA hybridisation to assess kallikrein gene expression in kidney tissue, in parallel with studies of renin gene expression. These and other powerful molecular biology techniques hold the promise of an answer to many of the unanswered questions regarding kallikrein and kinins, and their importance in health and disease.

List of publications describing results in this thesis

Cumming AD and Robson JS.
Urinary kallikrein excretion in glomerulonephritis and nephrotic syndrome.
Nephron, 1985; 39: 206-210.

Watson ML, Cumming AD, Lambie AT and Oates JA.
Urinary kallikrein and systemic prostacyclin synthesis during sodium chloride infusion in normal man. Clinical Science, 1985; 58: 537-543.

Cumming AD and Lambie AT.
Urinary kallikrein excretion in chronic renal failure; relationship to blood pressure and the acute effect of captopril.
Renal Failure, 1987; 10: 159-165.

Cumming AD, Driedger AA, McDonald JW, Lindsay RM, Solez K and Linton AL.
Vasoactive hormones in the renal response to systemic sepsis.
American Journal of Kidney Diseases, 1988; 11: 23-32.

Cumming AD, Kline R and Linton AL.
Association between renal and sympathetic responses to non-hypotensive systemic sepsis.
Critical Care Medicine, 1988; 16: 1132-1137

Cumming AD, Jeffrey S, Lambie AT and Robson JS.
The kallikrein-kinin and renin-angiotensin systems in nephrotic syndrome.
Nephron, 1989; 51: 185-191.

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